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THE ARTIFICIAL OPSONIZATION OF BACTERIA.

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L. REINER, with Oe. Fischer and H. Kopp (1929), found that tannic acid would sensitize red blood-corpuscles so as to allow hæmolysis by complement, and that it would also prepare red corpuscles or staphylococci for phagocytosis, acting as what may be termed an artificial opsonin. This opsonizing action appears to have been also simultaneously discovered by Freund (1929). Reiner's work attracted the attention of Neufeld (1929), who, with Etinger-Tulczynska, verified the previous results, and also tried a considerable number of other substances, chiefly salts, as substitutes for tannic acid. Complement hæmolysis of red corpuscles only took place when tannic acid was used, but many salts were found capable of acting as opsonins, either with red corpuscles, typhoid bacilli or pneumococcus Type I. Salts of aluminium, trivalent iron and chromium were particularly effective, and also the sulphate of tetravalent thorium. This led Neufeld to suggest that the opsonizing action depended on the presence of a tri- or tetravalent cation, since with salts of mono- and divalent metals (except lead) no opsonizing effect was produced. Neufeld emphasized the close connection existing between the opsonizing and agglutinating activities of these substances. There is a very obvious general correspondence in this regard which can be seen in his results, though occasionally the phagocytosis is weak when the agglutination is strong, and *vice versa*.

Reiner also noted the parallelism between opsonization and agglutination, but laid great stress on the alteration of the surface properties of red corpuscles and bacteria by the action of tannic acid, consequent upon the dehydrating effect which is part of the action of tannin. He based his views very largely on the theory of tanning due to H. R. Kruyt (1922, etc.) and his associates, and came to the conclusion that dehydration of the surfaces of corpuscles and bacteria is the chief effect of tannic acid when acting either as an opsonin, or

as a substitute for an antibody in complement hæmolysis. Reiner did not carry out any experiments with inorganic salts.

Neufeld, in addition to using a variety of salts, also tested the effect of alcohol and acetone on typhoid bacilli and pneumococcus Type I, the alcohol being used at absolute strength, and therefore able to exert a powerful dehydrating effect. In this experiment agglutination was obtained, but there was no phagocytosis when the bacteria were added to a suspension of leucocytes.

In considering the above results, it appeared to the writers that the substances which cause both agglutination and opsonization are, in general, those which are known to have tanning action. Agglutination alone can sometimes be effected by acid, either alone or buffered by sodium salt, as is shown by an experiment of Neufeld in which agglutination resulted from the action of a mixture of lactic acid and sodium lactate at pH = 4·2. However, the substances which produce both effects, *e. g.* tannic acid and salts of chromium, iron (ferric), aluminium, cerium and thorium are all capable of tanning, and many of them have a large industrial use for this purpose. On the other hand, salts of the alkali and akaline earth metals, also of zinc, magnesium, iron (ferrous) and nickel have very feeble tanning properties or none at all. In this connection it is interesting to recall Landsteiner's (1912) observations on silicic acid, which are discussed by Neufeld. Silicic acid was found to act as an agglutinin, to be capable of replacing the antibody in complement hæmolysis, and also able to act as an opsonin. Silicic acid is known to be a tanning agent. The writers were thus led to try further substances, with a view to establishing more firmly, if possible, the connection between tanning action and opsonization.

EXPERIMENTAL METHODS AND RESULTS.

The experimental technique was as follows : A suspension of staphylococci in saline was prepared by washing the bacteria from agar slopes ; 1 c.c. quantities of this suspension were centrifuged, and the deposited bacteria re-suspended in 1 c.c. quantities of the various solutions under test. The tubes were then allowed to stand 2–3 hours on the bench, after which the bacteria were separated and washed twice by centrifuging, and finally re-suspended in 1 c.c. of normal saline. Washed guinea-pig leucocytes were prepared as described by the writers in an earlier paper (1935). A few drops of the bacterial suspension were then added to 0·25 c.c. of saline together with one drop of leucocytic cream, and the mixtures incubated for $2\frac{1}{2}$ to 3 hours at 37° C. During incubation the tubes were gently shaken from time to time. Smears were then made and stained with Leishman's stain.

The substances tested for opsonic action were as follows, used in concentrations of from 0·1 to 1·0 p.c. :

(*a*) Chrome alum, chromic chloride, potassium bichromate reduced by sulphur dioxide, iron alum, ferric chloride, potash alum, aluminium sulphate.

(*b*) Tannic acid, also infusions or solutions of cutch (mangrove), quebracho, valonia, myrabolans, sumach, gambier, tara, mimosa, and chestnut wood.

(*c*) Gallic, acetic, lactic, sulphuric and picric acids.

(*d*) Catechin.

(*e*) Ferrous sulphate and ferrous ammonium sulphate; barium chloride and nitrate; cadmium chloride, acetate and sulphate; calcium chloride; lead acetate and nitrate; zinc sulphate; copper sulphate; magnesium sulphate; complex chromium oxalates; nickel sulphate; cobalt chloride.

(*f*) Formaldehyde.

Controls were carried out with saline and normal guinea-pig serum in every experiment. In only one instance was there any spontaneous phagocytosis observable, and also in one instance only did serum fail to produce very marked phagocytosis.

The substances under (*a*) are all very well-known inorganic tanning agents. The writers found, in harmony with Neufeld, that all of these acted as opsonins in inducing phagocytosis, and also, again like Neufeld, that the action is not perfectly regular. The iron salts were the most powerful and regular in their effects. The substances under (*b*) are the most widely used vegetable tannins, with the exception of tannic acid, which is of interest chiefly to pharmacists. All of these had opsonic action, though there was considerable variation amongst them in regard to the extent of the action. Of the acids under (*c*), picric acid is the only one known to have tanning action, and was the only one to show any opsonic activity. Its action, however, was very feeble, and shown only in partially neutralized dilute solutions. Catechin is a well known "non-tannin" found in gambier. This substance did not show opsonic activity. The salts under (*e*) have only very feeble tanning properties, or else are entirely devoid of such. The latter class includes barium, calcium and magnesium salts, none of which showed any trace of opsonic action. The salts of heavy metals have in some cases a little tanning action, but no opsonic activity was observed with these with the possible exception of nickel and cobalt salts. The behaviour of some chromium complex oxalates was interesting. These solutions contained no chromium in the ionic form, and were found to be devoid of both tanning properties and opsonic effect. It should be noted also that the non-tanning ferrous salts also failed to opsonize, in contrast to the ferric salts.

The last substance, formaldehyde, was tried by Neufeld, who found that it agglutinated at 10 p.c. strength, but did not opsonize. As this substance will, under carefully controlled conditions, act as a mild tanning agent, the writers investigated its action in several experiments. It was found, when used at pH = 8·3, to have a definite, though feeble, opsonizing effect.

To summarize the results obtained it may be stated (*a*) that all the substances which act as artificial opsonins have marked tanning properties, and (*b*) that the great majority of tanning substances, organic or inorganic, appear to be able to act as artificial opsonins. Some compounds such as formaldehyde or lead salts that possess feeble tanning properties have still more feeble opsonizing action.

If the substances tested were placed in order of diminishing tanning power this would also be the order of diminishing opsonic effect, but the opsonic effect becomes negligible or *nil*, whilst the tanning effect is still considerable.

DISCUSSION.

It has already been mentioned that Reiner, following Kruyt, ascribes the action of tannic acid to dehydration, whilst Neufeld, in considering salts, lays stress on the valency effect of the metallic ion. Neufeld does not deny the importance of dehydration, but nevertheless finds that absolute alcohol, a powerful dehydrant, fails to bring about opsonization. However, in the opinion of the writers, Kruyt's theory of tanning action is very incomplete. In actual fact tannin must of necessity combine irreversibly with protein, so as to produce a compound that cannot be broken down or decomposed by the action of water. Furthermore, the tannin-protein compound must be resistant, even in the moist state, to the action of putrefactive organisms. It will be seen, therefore, that for tanning action it does not suffice if a substance merely combines with a protein, as is the case with hydrochloric acid or sodium hydroxide—reagents which form hydrolysable compounds, *i. e.* which break down into their components by the continuous action of water. The complete chemical mechanism of tanning is by no means understood at present, but the most widely-accepted view, shared by the writers, is that the organic tannins, in the first instance, combine with ionized amino-groups of proteins to form non-hydrolysable or " insoluble " bodies. Schematically this may be represented as follows :

$$R\begin{cases}NH_3^+\\COO^-\end{cases} + HT \rightarrow R\begin{cases}NH_3T\\COOH\end{cases},$$

where HT represents the tannin, always a weak acid. The same scheme will serve for the combination of hydrochloric acid, substituting HCl for HT, but there is a great difference in the properties of the resulting compound, just as salts of the same metal with different acids often have very divergent properties in respect of solubility. The mechanism of chrome tanning differs in that the combination of the metallic tanning compound is through the —COOH groups of the protein, but the end-result is again the production of a non-hydrolysable compound.

Since we are here concerned only with weak acids or bases, a non-hydrolysable " insoluble " compound must also be non-ionized, and compounds of proteins and tannins differ fundamentally in this respect from gelatin hydrochloride, which is very extensively ionized :

$$G\begin{cases}NH_3Cl\\COOH\end{cases} \rightarrow G\begin{cases}NH_3^+\\COOH\end{cases} + Cl^-.$$

It has been shown by Procter and Wilson (1916) that the swelling of solid proteins in acids (or alkalis) is directly due to the ionization of the protein-acid compound, and that any treatment of a swollen protein which diminishes the ionization must cause a diminution of the swelling, *i. e.* dehydration. On this view, therefore, dehydration is a direct consequence of tanning action, brought about by the substitution of non-ionized for ionized groups, but it is not any more than this. Proteins can be dehydrated in many ways, *e. g.* by alcohol, without any tanning action taking place.

There are thus two steps in the action of tannins on proteins: first the production of non-ionized, non-hydrolysable or " insoluble " compounds by the immediate combination of tannin and protein, and secondly the consequent

dehydration of the system. The new compound must necessarily have different properties from those of the original protein. If ionized groups are replaced by un-ionized, there must be a corresponding diminution in electrical charge, and an increased tendency to agglutination. Actually, as has been mentioned above, agglutination almost always accompanies the action of substances which act as artificial opsonins, and this appears to the writers to be a very natural consequence of tanning action, which merits closer study.

With regard to the mechanism of the opsonization by tannins, it is not possible to offer more than the general statement that the substitution of non-ionized for ionized or uncharged for charged groups in the surface of the bacterium must lead to radical changes in all the surface properties, and that the new state is more favourable to phagocytosis than the original state. It is nevertheless interesting, and no doubt important, to find that so many substances apparently very diverse chemically have two properties in common, namely tanning action and the power of opsonization—properties which at first sight appear to have little relation with one another.

SUMMARY.

The work of Reiner and of Neufeld has been followed up and extended. In addition to tannic acid and the various salts studied by the previous workers, a large range of other substances has been tested for opsonic action on staphylococcus. This range includes many vegetable tannins, salts of chromium, iron, aluminium, lead, zinc, copper, magnesium, calcium, barium, cadmium, nickel and cobalt, and also gallic, acetic, lactic, sulphuric and picric acids, formaldehyde and catechin. A definite parallelism has been found between tanning action and opsonic activity, and it is considered that the irreversible chemical changes characteristic of tanning are responsible for the changes in physical properties which make phagocytosis possible. Dehydration is an invariable consequence of tanning action, but the writers do not consider it to be the primary factor. The substances which show opsonic activity almost always possess agglutinating power—a property which a tannin would be expected to show on theoretical grounds.

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REFERENCES.

FREUND, J.—(1929) *Proc. Soc. Exp. Biol. N.Y.*, **26**, 876.
GORDON, J., AND THOMPSON, F. C.—(1935) *Brit. J. exp. Path.*, **16**, 101.
KRUYT, H. R., AND DE JONG, H. G. B.—(1922) etc., *Kolloidzschr.*, **31**, 338.—*Rec. Trav. chim. Pays-Bas*, **42**, 437 ; **46**, 727.—*Collegium*, 1925, 341, and 1929, 545.
LANDSTEINER, K., AND ROCK, R.—(1912) *Z. ImmunForsch.*, **14**, 21.
NEUFELD, F., AND ETINGER-TULCYZNSKA, R.—(1929) *Zbl. Bakt.*, I Abt., Orig., **114**, 252.
PROCTER, H. R., AND WILSON, J. A.—(1916) *J. chem. Soc.*, **109**, 307 and 1327.
REINER, L., AND FISCHER, Oe.—(1929) *Z. ImmunForsch.*, **61**, 317.
REINER, L., AND KOPP, H.—(1929) *Ibid.*, 397.

EVIDENCE REGARDING THE MECHANISM OF ELIMINATION OF 1 : 2-BENZPYRENE, 1 : 2 : 5 : 6-DIBENZANTHRACENE, AND ANTHRACENE FROM THE BLOOD-STREAM OF INJECTED ANIMALS.

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In the course of biochemical studies of fowl sarcomata induced by chemical carcinogenic agents Chalmers (1934) noted the absence of recoverable 1 : 2 : 5 : 6-dibenzanthracene in tumours induced by this substance dissolved in fatty media, and, from subsequent experiments, found that 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard, egg-yolk fat or chicken fat, rapidly disappeared from the site of intramuscular injection, though its subsequent fate could not be determined at the time. Analogous experiments with 1 : 2-benzpyrene have shown that this substance also disappears rapidly from the site of intramuscular injection.

The author's experiments with fowls injected intramuscularly with 1 : 2 : 5 : 6-dibenzanthracene, dissolved in lard, egg-yolk fat and chicken fat (Peacock, 1935) indicated that the carcinogenicity of this substance seems to depend partly upon the local reaction to the medium used as a solvent; 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard was more carcinogenic than the same quantity dissolved in egg-yolk fat, while with chicken fat as a solvent it gave negative results. Attempts to trace the fate of dibenzanthracene and benzpyrene after intramuscular injection in fatty solvents have not given satisfactory results in our hands, probably owing to the difficulty associated with the extraction of tissues and excreta of the injected birds and the presence of fluorescent bodies and pigments, which tend to mask the characteristic absorption and fluorescence spectra of these carcinogens. From similar observations of Berenblum and Kendal (1936) on mice, it seems that these animals can also destroy or eliminate 1 : 2 : 5 : 6-dibenzanthracene within a period far short of that required for tumour growth to become recognizable, though traces of dibenzanthracene were found to remain encapsulated and unchanged for prolonged periods in some cases. These authors also found that the medium in which the dibenzanthracene was injected intraperitoneally in mice affected both the rate of disappearance and the incidence of tumours; colloidal dibenzanthracene tended to become encapsulated and to remain partially unabsorbed, while dibenzanthracene dissolved in lard was more

rapidly eliminated; the tumour incidence was higher after injections of colloidal dibenzanthracene injections than of dibenzanthracene dissolved in lard. Berenblum and Kendal also injected fowls intravenously, giving 20 mg. of dibenzanthracene in colloidal suspension in thirty weekly injections. None of these birds developed tumours, though they were kept under observation for a period of two years. They were then killed and extracted with benzene and examined spectroscopically; no dibenzanthracene was detectable in any of the extracts.

Boyland and Burrows (1935) attempted to induce tumours in rats and mice by causing local irritation and tissue reaction with powdered silica introduced subcutaneously, followed by multiple subcutaneous and intraperitoneal injections of colloidal 1 : 2 : 5 : 6-dibenzanthracene, but obtained tumours only at the sites of injection of the latter substance. They also injected colloidal 1 : 2 : 5 : 6-dibenzanthracene intravenously into rabbits, using silica as a connective-tissue irritant and chloroform as an epidermal irritant, but failed to produce any tumours by these means.

From consideration of these experimental results it appears probable that dibenzanthracene is relatively harmless when circulating in the blood-stream, and that its locally carcinogenic activity is conditioned in some way by the local tissue response to the inoculum, part of which may be due to the medium used as a solvent. In the experiments to be described the fate of dibenzanthracene, benzpyrene, and anthracene, following intravenous injection of colloidal suspensions of these substances in water, has been carried a stage further. Rabbits, guinea-pigs and fowls were used, but principally the latter, partly because we have considerable data relating to tumour induction by carcinogens in these birds, and partly for technical reasons, as will be seen later. As 1 : 2-benzpyrene is more brilliantly fluorescent than 1 : 2 : 5 : 6-dibenzanthracene, it has been found more convenient to use this substance, and it has been used in the majority of the experiments. The following descriptions, however, apply equally well to dibenzanthracene and anthracene colloid, except where otherwise stated.

EXPERIMENTAL.

When a film of colloidal 1 : 2-benzpyrene is examined microscopically by ultra-violet illumination, either by the dark-ground method described by the author in a different connection (Peacock, 1934), or more simply by direct ultra-violet illumination in a darkened room,* it is seen that every particle of benzpyrene can be clearly recognized by its brilliant yellow fluorescence. The effect is very reminiscent of the Milky Way on a clear starry night, and the Brownian movement of the particles gives rise to a twinkling effect that further enhances this analogy. Quantities of 1 : 2-benzpyrene, of the order of 10^{-10} mg., are very easily recognized in a single field under a 4 mm. objective by this simple method. If we take the total blood volume of a fowl of average size, say 1300 g., as approximately 100 c.c., the resulting concentration of 1 : 2-benzpyrene in the blood following an intravenous injection of 5 c.c. of 0·01

* Demonstrated at the meeting of the Society of Pathology and Bacteriology, St. Bartholomew's Hospital, London, January, 1936.

p.c. colloid, assuming that the blood acts simply as a diluting medium, will be 0·005 mg. per c.c. Assuming the volume of a wet blood film to be about 1 c.mm., such a film would contain 5×10^{-6} mg. of benzpyrene—a quantity very easily recognizable by the method in question. Normal blood of birds (fowl, pigeon) or mammals (man, rabbit, guinea-pig) has been examined on many occasions under ultra-violet illumination, but has never been found to contain fluorescent particles. (Occasional fragments of hair, feathers or epithelial scales are entirely different in appearance and size from the particles in colloidal suspensions of benzpyrene.)

Fowls of from 1000 to 1800 g. were injected in the right alar vein with 5 c.c. of 0·01 p.c. benzpyrene colloid. The injection was made slowly, taking about one minute, and, immediately after withdrawal of the injection needle, a drop of blood taken from the opposite alar vein was examined as a wet film before clotting occurred. Films prepared in this way show enormous numbers of fluorescent particles in Brownian movement corresponding exactly in appearance with mixtures of blood and colloid *in vitro*. Similar films prepared from blood collected at intervals of a few minutes showed two effects within about a quarter of an hour of injection. Firstly, there is an obvious and rapid diminution in the number of fluorescent particles per microscopic field, and, secondly, there is a loss of Brownian movement and an increase in size of the fluorescent particles. Within half an hour, as a rule, very few fluorescent particles can be seen in a single drop of blood, and after one hour it is exceptional to find even a single particle. Similar experiments on rabbits and guinea-pigs gave similar results. Equivalent quantities of 1 : 2 : 5 : 6-dibenzanthracene and of anthracene colloid, similarly injected, disappear from the circulating blood in a similar manner to that described for 1 : 2-benzpyrene. *In vitro* citrated blood does not destroy 1 : 2-benzpyrene colloid to a significant extent when incubated for half an hour at 37° C. The evidence, therefore, points to destruction, fixation, or elimination of the colloids following intravenous injection. It was thought probable that phagocytosis might play a part in the mechanism of this disappearance, and this possibility was tested, as follows :

Blood-films were made at intervals of a few seconds to a few minutes from fowls injected 15–30 minutes previously with 1 : 2-benzpyrene colloid. These were allowed to dry and were examined unstained for fluorescence. The fluorescent particles were then mapped. The films were stained (Leishman), and the same fields were examined and the position of the white cells was mapped. Comparison between maps of identical fields of the stained and unstained films showed no correspondence between the positions of the fluorescent particles and those of the white cells or platelets. It may be mentioned here that alcoholic stains destroy the fluorescence by dissolving the hydrocarbons. Similar experiments with rabbits and guinea-pigs have given analogous results.

In an attempt to pursue the fate of these colloidal hydrocarbons, fowls, rabbits and guinea-pigs were injected intravenously with 1 : 2-benzpyrene colloid, as described, and the blood fluorescence was examined at 5-minute intervals until only an occasional fluorescent particle could be seen. The animals were then killed, and frozen sections of the organs were examined by

ultra-violet illumination. Preliminary experiments on these lines gave little indication of the fate of benzpyrene colloid, and, in an endeavour to locate the effective mechanism, three rabbits were splenectomized, one had both renal arteries tied, one had the hepatic artery tied, three were injected with trypan blue, two with indian ink, and one with trypan blue and indian ink, some hours before intravenous injection of the 1 : 2-benzpyrene colloid. None of these procedures prevented the disappearance of the benzpyrene colloid from the circulating blood, though it was noticed that in animals that had recently undergone operations under anæsthetics (" Evipan " or $CHCl_3$), and whose metabolism was no doubt diminished, the rate of disappearance was somewhat slower than in active normal animals. Perfusion of two recently killed intact rabbits, with 50–100 c.c. of 0·01 p.c. benzpyrene colloid, followed by perfusion with normal saline and examination of frozen sections, showed fluorescent particles in liver, spleen, choroid plexus and appendix.

The perfusion of individual organs was next tried. In the case of the kidney, one kidney was removed from a rabbit for control, and the remaining organ was perfused with 1 : 2-benzpyrene colloid, through the renal artery. Frozen sections revealed colloid in the blood-vessels of the perfused organs, but no other difference from the control. The livers of two rabbits were perfused after tying-off one lobe as a control. Here, owing to difficulty of access in the living animal, a cannula was tied into the descending aorta, and the abdominal viscera were perfused in one experiment ; in another the liver was perfused through the portal vein. A study of frozen sections showed that the perfused liver was capable of dissolving the colloid. Control sections, under ultra-violet illumination, showed the presence of small intra-cellular globules of greenish fluorescence. Sections of perfused liver showed not only 1 : 2-benzpyrene colloid in the lumina of the vessels, but the normally present globules now showed a lavender-violet fluorescence similar to that of 1 : 2-benzpyrene in dilute solution. This effect was more marked in frozen sections that had stood for twenty-four hours. Repetition of this experiment in fowls and guinea-pigs gave similar results. As fat stains were not always taken up by the fluorescent globules seen, it seemed possible that solution of the 1 : 2-benzpyrene in fats or lipoids in the liver was a first-stage in the disposal of this substance ; the bile was, therefore, examined as a possible vehicle for its elimination. *In vitro*, tests showed that bile is a remarkably good solvent for 1 : 2-benzpyrene, even at room temperature, and will dissolve this compound from ten times its volume of 0·01 p.c. benzpyrene colloid, to give a brilliantly violet fluorescent solution. In ordinary light the bile appeared unchanged. 1 : 2 : 5 : 6-dibenzanthracene and anthracene do not dissolve in bile so readily as 1 : 2-benzpyrene, but will dissolve in warm diluted bile.

Here, then, was a possible route for elimination, since the quantity of bile normally present in an average fowl's gall-bladder is 1–2 c.c.—more than enough to dissolve the whole of the 1 : 2-benzypyrene injection in 5 c.c. of colloid. In six fowls—two cocks and four hens—the gall-bladder was exposed under " evipan " anæsthesia, and a short, wide-mouthed glass cannula was tied in. The cannula was connected with a short piece of sterile valve tubing, which was brought out through a stab wound in the abdominal wall. The rubber tube was plugged with a glass stud, and, as soon as the abdomen had been closed

completely, a sample of bile was withdrawn by inserting the nozzle of a small glass syringe and exerting gentle pressure on the abdomen of the bird. This was sufficient, usually, to expel the gall-bladder contents into the syringe. Suction, by means of the syringe, was found to be liable to cause collapse of the gall-bladder wall into the cannula, with consequent damage to the mucosa, resulting in an inflammatory condition and blocking of the cannula. The glass stud was attached by a suture to the abdominal skin, and the tube was marsupialized for about 1 cm. to allow of subsequent easy manipulation, while protecting it from interference by the bird. Cholecystostomy was similarly performed on four rabbits and on one guinea-pig, but, although all stood the operation well, no bile could be withdrawn, and after repeated unsuccessful attempts the animals were killed, and the gall-bladders found to be empty and contracted on to the cannula in each case. For these animals it was found more satisfactory to inject benzpyrene colloid intravenously, and then, at various time intervals, to expose the gall-bladder, under anæsthetic, and aspirate some of the bile with a syringe and fine needle. This procedure does not appear to interfere seriously with the normal functioning of the gall-bladder. Fowls tolerate the biliary fistula very well, and there is little or no interference with their appetite following the operation. During the day of operation bile samples were collected at intervals, and it was found possible to obtain 0·5 c.c. to 2 c.c. every hour, or even more frequently. The following day 5 c.c. 1 : 2-benzpyrene colloid was injected into each bird, and bile samples were withdrawn at intervals and examined. It was at once apparent that the bile, shortly after intravenous injection of 1 : 2-benzpyrene colloid, was brilliantly fluorescent, the fluorescence being violet and similar to that of 1 : 2-benzpyrene in solution in bile. Similar results were obtained with rabbits and guinea-pigs (see Tables I and II). Fluorescence spectra of such fluorescent bile samples, however, do not show the characteristic bands of benzpyrene.

From reference to the Tables it will be seen that excretion commenced probably immediately after injection and was well established within 15 minutes, though the return to the normal brownish fluorescence of the bile took about 6 hours. Anthracene colloid gives rise to a fluorescent substance in the bile somewhat similar to that from 1 : 2-benzpyrene, but the fluorescence is less marked. So far, 1 : 2 : 5 : 6-dibenzanthracene has not given positive evidence of elimination in the bile. Fluorescence spectra of samples of bile from two fowls, injected intravenously with colloidal 1 : 2-benzpyrene (5 ml. 0·01 p.c.), show general fluorescence in the blue-violet region of the spectrum without the characteristic bands of 1 : 2-benzpyrene, but with two ill-defined bands, with maxima at, approximately, 4200 and 4550 Å.U. This suggests that the liver not only excretes, but chemically alters this compound. The subsequent fate of the 1 : 2-benzpyrene, or its fluorescent derivative, after excretion in the bile, has not yet been fully worked out, but some experiments in which the intestinal contents at different levels have been extracted and examined suggest that the fluorescent substance excreted in the bile is destroyed in its passage down the gut, possibly by digestive juices or by bacterial action. Unchanged benzpyrene in solution has been observed in the excreta from two fowls injected intravenously with benzpyrene colloid ; in one bird the excreta

TABLE I.—*Recovery of Fluorescent Bodies from Biliary Fistulæ, following Intravenous Injection of Fluorescent Polycyclic Hydrocarbons in Colloidal Suspension.*

Fowl No.	Substance injected.	Fluorescence of bile at different time intervals after injection.											Later observations.
		15 min.	30 min.	1 hour.	1½ hours.	2 hours.	2½ hours.	3 hours.	3½ hours.	4 hours.	4½ hours.	5 hours.	
1293	B-P.C. 5 c.c.	..	..	..	..	..	..	..	..	+	..	..	Fistula was blocked. Bird killed at 4 hours.
G. 1685	B-P.C. 2·5 c.c.	+	+++	++	..	+	+	+	..	..	..	..	..
1306	B-P.C. 5 c.c.	..	..	++	+	+	..	..	..	+	+	..	— at 6 and 24 hours.
1271	B-P.C. 4 c.c.	+	..	..	..	..	..	..	..	..	..	..	Residual bile + (faint) when killed at 20 hours.
1561	B-P.C. 5 c.c.	..	..	..	..	++	..	..	..	..	..	..	..
1280	B-P.C. 9 c.c.	..	+++	+++	++++	..	..	++	+	..	..	..	— at 20 hours.
1280	B-P.C. 10 c.c.	..	+++	..	..	..	..	..	..	+	..	..	— at 48 hours.
1306	D-B.C. 5 c.c.	..	..	−	+?	..	..	..	..	..	..	..	Fluorescence very faint.
982	D-B.C. 10 c.c.	..	−	−	−	−	..	−	..	−	−	..	..
1561	D-B.C. 5 c.c.	..	..	+?	..	..	..	..	..	..	..	..	..
1562	D-B.C. 5 c.c.	..	..	+?	..	..	..	..	..	..	..	..	..
1037	A.C. 5 c.c.	..	..	+	..	+	..	..	−	..	−	−	..
1521	A.C. 5 c.c.	..	..	..	+	..	..	..	..	..	..	..	Killed 1½ hours.
1274	A.C. 5 c.c.	..	++	..	..	++	..	+	..	+	..	+	Killed 24 hours; bile −.

B-P.C. = 0·01 p.c. 1 : 2-benzpyrene colloid, *ex* pyridine dialysed against distilled water.
D-B.C. = 0·01 p.c. 1 : 2 : 5 : 6-dibenzanthracene colloid, *ex* pyridine dialysed against distilled water.
A.C. = 0·06 p.c. anthracene colloid, *ex* pyridine dialysed against distilled water.
+? = Doubtful fluorescence.
Normal bile collected from each bird before injection gave negative fluorescence.

TABLE II.

Animal No.	Substance injected.	Fluorescence of bile at different time intervals after injection.						Later observations.	
		30 min.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	Bile fluorescence.	Urine fluorescence.
R. 243	B-P.C. 5 c.c.	..	++	..	..	..	++	..	..
R. 244	B-P.C. 10 c.c.	..	..	..	..	..	++	..	Blue +.
R. 245	B-P.C. 10 c.c.	..	..	..	..	..	++	..	..
R. 248	B-P.C. 5 c.c.	..	..	..	..	++	..	— at 18 hours	..
R. 248	B-P.C. 5 c.c.	..	+	..	..	..	..	Masked by hæmorrhage	..
R. 249	B-P.C. 5 c.c.	..	..	..	..	..	+	..	..
G-P. 3	B-P.C. 1 c.c.	+?	+	..	..	—	..	..	..
R. 246	D-B.C. 5 c.c.	..	..	..	..	..	—	..	..
R. 247	D-B.C. 5 c.c.	..	..	..	..	..	+?	Bright green	
R. 251	D-B.C. 5 c.c.	..	+?	..	—	..	..	Bright ochre-brown	Normal blue.
R. 252	D-B.C. 5 c.c.	..	..	..	..	+?	..	Brown	„
R. 243	A.C. 5 c.c.	..	+?	..	..	..	..	Brownish	Blue-violet +.
R. 250	A.C. 5 c.c.	+	..	..	..	..	..	..	Normal light-blue.
G-P. 4	A.C. 2 c.c.	..	+	..	..	..	..	..	Blue-violet ++.

R. = Rabbits. G.-P. = Guinea-pigs.
B-P.C. = 1 : 2-benzyrene colloid, 0·03 p.c. *ex* acetone.
D-B.C. = 1 : 2 : 5 : 6-dibenzanthracene colloid, (*a*) 0·01 p.c. *ex* pyridine; (*b*) 0·05 p.c. *ex* acetone.
A.C. = Anthracene colloid 0·06 p.c. *ex* pyridine.

passed one hour after injection, and in the other the pooled excreta for 48 hours after injection, both showed the characteristic bands of benzpyrene. This seems to suggest that unaltered benzpyrene may be excreted in the bile despite the positive evidence that the fluorescence of the bile following injection of benzpyrene is not entirely due to this substance. In rabbits it has been found that the urine contains an ether-soluble substance of blue fluorescence, not normally present, following intravenous injection of benzpyrene.

DISCUSSION.

The similarity of behaviour of colloidal 1 : 2 : 5 : 6-dibenzanthracene, 1 : 2-benzpyrene, and anthracene, following intravenous injection in fowls and rabbits, as regards their rate of disappearance from the blood-stream, suggests that they are subject to a similar mechanism of elimination. In the case of benzpyrene and anthracene, the blue fluorescence of the bile, shortly after intravenous injection of the colloid, increasing in intensity as the fluorescent particles in the circulating blood decrease in number, and lasting only for a few hours after injection, points to the elimination of these two substances by the liver. Frozen sections of perfused livers, examined by ultra-violet illumination, further strengthen this conception. The absence of similar fluorescence in the bile, following the injection of similar quantities of 1 : 2 : 5 : 6-dibenzanthracene colloid, suggests that this substance, if subject to the same mechanism of elimination, is converted into a non-fluorescent derivative.

The negative results of examination of excreta from animals injected with these colloids suggests that after elimination in the bile further chemical action occurs in the bowel, though, as mentioned, it is possible to obtain evidence of unchanged benzpyrene in the fæces in some cases. This may be due to supersaturation of the normal eliminating mechanism by a sudden access of the colloid, following intravenous injection. Whereas, after intramuscular injection, absorption being slower, the elimination and destruction of the carcinogen is complete before the excreta are voided.

It is possible that some water-soluble derivative of benzpyrene is formed in the liver, or is re-absorbed from the gut and excreted by the kidney, as the urine of rabbits, after intravenous injection of benzpyrene colloid, has been found to contain an ether-soluble fluorescent substance not normally present.

Evidently there are many points in connection with this work that require further elucidation, but the mechanism described is sufficiently well established to justify publication. Moreover, although positive evidence of the mechanism of elimination of 1 : 2 : 5 : 6-dibenzanthracene is lacking, there is clear evidence that dibenzanthracene colloid is rapidly eliminated from the circulating blood, and this may account for the failure to induce tumours by intravenous injection of this colloid.

It is certainly unlikely that 1 : 2-benzpyrene colloid would give positive results in similar experiments. It seems probable that many other hydrocarbons would be eliminated in the bile, following intravenous injection.

The concentrating action of the gall-bladder must tend to subject this organ to a more intense action of benzpyrene or its derivatives than would be the case for the other organs, and it is interesting to realize that in this mechanism

we have an opportunity of subjecting a secreting epithelium to repeated application of a chemical carcinogen, or its derivatives, in considerable concentration, without the necessity for any local mechanical interference.

SUMMARY.

1. Colloidal 1 : 2-benzpyrene, 1 : 2 : 5 : 6-dibenzanthracene and anthracene disappear rapidly from the circulating blood, following intravenous injection, in rabbits, guinea-pigs and fowls.

2. The white cells of the blood are not responsible for this disappearance.

3. Frozen sections of various organs show that the liver contains intracellular globules of lavender fluorescence, following intravenous injection of benzpyrene colloid.

4. Bile samples obtained by cholecystostomy show blue-violet fluorescence shortly after intravenous injection of benzpyrene or anthracene colloid, but not after dibenzanthracene colloid.

5. The fluorescence of cholecystostomy bile, following benzpyrene colloid injection intravenously, is not due to this substance, but to a fluorescent derivative, or derivatives, showing ill-defined fluorescent bands with maxima at, approximately, 4200 and 4550 Å.U.

6. This substance has not been recovered from excreta, but unchanged benzpyrene in solution has been found occasionally in the excreta, after intravenous injection of large amounts of the colloid.

7. In rabbits the urine, after intravenous injection of benzpyrene, dibenzanthracene, and anthracene colloids, contains an ether-soluble substance, or substances, of blue fluorescence not normally present. Fluorescence spectra of extracts of such urine show no characteristic bands.

I am indebted to Dr. L. Woodhouse Price for making some of the frozen sections, and to Mr. J. G. Chalmers for recording the fluorescence spectra.

REFERENCES.

Berenblum, I., and Kendal, L. P.—(1936) *Biochem. J.* (in press).
Boyland, E., and Burrows, H.—(1935) *J. Path. Bact.*, **41**, 231.
Chalmers, J. G.—(1934) *Biochem. J.*, **28**, No. 4, 1934.
Peacock, P. R.—(1933) *J. Path. Bact.*, **36**, 141.—(1934) *Brit. J. phys. Med.*, **8**, 173.—(1935) Leeuwenhoek-Vereninging, Amsterdam.

BEHAVIOUR OF LOUSE-BORNE (EPIDEMIC) AND FLEA-BORNE (MURINE) STRAINS OF TYPHUS *RICKETTSIA* IN TISSUE CULTURES.

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THE demonstration by Nigg and Landsteiner (1932), confirmed by Kligler and Aschner (1933), that typhus *Rickettsia* can be grown in tissue cultures in flasks (Maitland medium) and maintained *in vitro* for several generations, has opened the possibility of a more systematic study of the nature of these organisms. As in the case of ordinary bacteria, cultivation *in vitro* is important as a means of advancing our knowledge on the nature of the virus.

Kligler and Aschner (1934, 1935) have shown that killed vaccine prepared from cultures may, on the one hand, serve as an efficient immunizing agent, and, on the other, assist in the differentiations of the various types of *Rickettsia*. It is clear, however, that the technique of the cultivation itself has to be refined and improved if constant and uniform results are to be obtained. The experiments reported below were designed both to improve the technique of cultivation and to throw light on the biological characters of the virus.

MATERIALS AND METHODS.

Strains.—The experiments were carried out with two strains of typhus *Rickettsia*. One strain was isolated from a case of typhus in Europe, and was kindly furnished us by Dr. Breinl; the other we owe to the generosity of Dr. Lépine, and was isolated by him from wild rats in Greece. The European strain has been maintained in our laboratory by guinea-pig brain passage since 1930, while the rat strain has been maintained since 1932 by guinea-pig passage of tunica and spleen.

The reactions produced by the two strains have remained constant. The injection into guinea-pigs of 1 c.c. of a 1 : 30 brain emulsion in saline of the European virus produces a characteristic rise in temperature on the 6th or 7th day after infection. The fever lasts 5 to 6 days, the temperature always rising to 40° C. or over. At no time have changes in the scrotum or scrotal swelling been observed, nor have *Rickettsia* been found in smears and preparations from the tunica vaginalis. In these respects this strain differs, apparently, from the Nicolle Tunis strain, which at times produces scrotal

lesions and in which, according to Nigg and Landsteiner (1932), *Rickettsia* are found occasionally in smears of the tunica.

The murine strain has also given fairly constant reactions. The injection of 1 c.c. of a 1 : 30 suspension of testicles and spleen in saline produces a rise in temperature after an incubation period of 4–5 days. The period of fever lasts only about 5 days, as noted by Lépine ; the temperature is saddle-back rather than continuous, and generally lower than that produced by the European strain. As a rule, the scrotum of the infected guinea-pigs shows the characteristic scrotal reddening and swelling on about the 5th day of the infection, and *Rickettsia* can be readily found in smears of the tunica vaginalis.

The two strains may, therefore, be considered typical representatives of the two types.

Media.—The cultivation was carried out in a modified Maitland medium. Nigg and Landsteiner, the first to use the Maitland medium for the cultivation of *Rickettsia*, made their cultures by bringing normal tunica tissue in contact with a suspension of *Rickettsia* from an infected tunica. This method is effective only when the infected tunica contains a large amount of organisms. When the tissue is only slightly infected, as is the case in the louse-borne virus, the cultures are negative. In our work we have found that much more constant results could be obtained by using as tissue for initial cultures the tunica of infected guinea-pigs. This procedure greatly simplifies the technique and, moreover, often gives positive cultures even when no *Rickettsia* can be found before culturing. The scanty infection initiated *in vivo* progresses in the cultures *in vitro* until a large number of the cells become heavily infected. One thus obtains a tissue invasion with *Rickettsia* never obtained in the guinea-pig, and like that found in the insect vector. Subcultures are easily made from these active rich cultures.

Method of cultivation.—The technique finally adopted was as follows : The testes of an infected guinea-pig are removed aseptically into a sterile petri dish on the 1st day of fever. A longitudinal incision is made in the testicle and the tunica peeled off with a sterile scalpel. It is important to remove all fat and other tissue, because their presence adds nothing to the culture, and interferes in the subsequent microscopic examination for *Rickettsia*. In addition to the tunica we also used the mesorchium, a thin membrane between the tunica and vas deferens. This membrane, because of its thinness and freedom from connective tissue, is particularly useful for microscopic examination, for which it can be used *in toto*.

After being freed of extraneous tissue and fat, the tunica and mesorchium are placed in a drop of serum and thoroughly minced with fine sterile scissors. This material is divided into 6 or 8 equal parts, each of which is transferred with a platinum spatula into a culture flask.

The culture flasks used by us are small Erlenmeyer bottles, of 25 c.c. capacity. To each flask are added 3·0 c.c. of the culture fluid, consisting of 2·5 c.c. Tyrode solution and 0·5 c.c. guinea-pig serum. We always add 5 c.c. of phenol red to a litre of Tyrode solution because the indicator shows what transpires in the culture. After inoculation with the tissue, the flasks are closed with sterile rubber stoppers and incubated at 30° C.

The microscopic examinations are usually made after 8–10 days. Small

bits of tissue are removed with a loop, placed on a clean slide and teased out until spread very thin. The preparation is dried in the air, fixed with methyl alcohol and stained with Giemsa. The microscopic picture of an endothelial cell infected with *Rickettsia* is so characteristic that the identification presents no difficulties.

For subcultures a few pieces of infected tissue are removed into a sterile hollow glass slide containing a tiny drop of guinea-pig serum. The tissue is then thoroughly minced with fine-pointed scissors. The tunica from a normal guinea-pig is then spread out in the *Rickettsia* suspension so as to obtain maximum contact and allowed to soak 10–15 minutes. The tissue is then cut up, divided and transferred to fresh culture flasks, as in the primary cultures.

Appearance of cultures.—The condition of the tunica cell is the determining factor in the growth of the *Rickettsia*. If for some reason the cells are injured, then no growth occurs. If the cells remain intact, then the growth is regular and constant.

Macroscopically one can soon tell whether the culture is in good condition. The indicator shows the changes occurring in it. Normally the serum-Tyrode changes slowly from a mildly alkaline (pH 7·4–7·6) reaction to a neutral one. If the culture is contaminated, the reaction becomes distinctly acid; if there happens to be a leak in the stopper, that is, if the flask is not properly closed, the reaction becomes more alkaline, due to the loss of CO_2. In either case growth fails to develop. Another indication is the appearance of the tissue in the culture. In properly growing cultures the fluid remains perfectly clear, and the fragments of tissue assume a characteristic appearance of small, round clumps or granules in place of the flat pieces first inoculated. In some cultures one finds a sort of halo consisting of amœboid extensions of cells around the tissue fragments adhering to the bottom of the flask.

Appearance of Rickettsia *in cultures.*—On microscopic examination one finds *Rickettsia* in large numbers, both inside and outside the cells. The free organisms have been released by disintegrated cells. Often more than half of the endothelial cells are infected, the organisms filling the plasma so completely that with Giemsa stain the nucleus is barely visible. However, even in the heaviest infection no organisms were noted in the nucleus (Figs. 1 and 3).

In order to obtain a clearer idea of the distribution and growth of *Rickettsia* in the tissue, a number of individual tissue granules were fixed by the methods of Bouin or Regaud, embedded, sectioned and stained with Giemsa. It appeared that the *Rickettsia* form infected foci in the tissue. The infection is most intense in the centre of the focus, where the cells are, as a rule, necrotic. Around this circle there are fewer and fewer infected cells, the peripheral cells being hardly infected at all. It is particularly in these slightly infected cells that one finds the long thread form of *Rickettsia*; in heavily infected cells one finds only the characteristic coccoid forms. One gathers the impression that these filaments carry the extension of the infection from cell to cell, and that only later, when the cell is more heavily infected, do they break up into the typical coccoidal forms. This impression is supported by the fact that more thread forms are found in young cultures, and also that in the louse the pleomorphic forms appear in the early stages of the infection.

Nigg and Landsteiner found in their tissue cultures that the Nicolle strain

grew in thread forms in the first generation, while the murine strain did not. We could not observe any difference in this respect. Both the rat and louse-borne strains gave thread forms in young cultures and coccoid forms in old ones (Fig. 2).

BIOLOGY OF THE *RICKETTSIA*.

*Suitable time for cultures.**—These experiments were designed to indicate at what stage of the infection *Rickettsia* are most abundant, as well as the length of time they persist in the tissues. For this purpose the infected testicles were removed, and cultures made at different stages of the infection. In guinea-pigs infected with the rat strain the earliest time *Rickettsia* could be demonstrated in cultures was the 4th day; the richest cultures were obtained on the 6th day; on the 12th day of infection the cultures were as a rule negative.

The louse-borne strain gave totally different results. At the beginning of the investigations the cultures of the tunica were negative, and cultures could only be initiated by using louse *Rickettsia* (Kligler and Aschner, 1933). Subsequently we found that while the tunica was either negative or only slightly infected, *Rickettsia* could constantly be demonstrated from cultures made from the mesorchium. Repeated experiments showed that on the 4th day after infection the cultures were negative; on the 5th, 6th and 8th day the cultures of the mesorchium were positive and those of the tunica negative; on the 7th day an occasional culture of the tunica also contained a few *Rickettsia*. After the 8th day the results were no longer uniform; in one case a positive culture of the mesorchium was obtained on the 12th day; in others cultures on the 11th day were already negative.

It may be stated, therefore, that in animals infected with the murine strain the tunica as well as the mesorchium are invaded early in the infection, that the *Rickettsia* persist throughout the febrile period, and that the optimum time for culture is on the 6th day of infection. On the other hand, in animals infected with the louse-borne or European strain, the tunica is rarely invaded, and then only on the 7th day of infection; the mesorchium is infected as early as the 5th day, the optimum time for culture being the 7th day. The two strains differ, therefore, in the rapidity and extent of their invasiveness in the tunica membrane.

In rats infected with the murine strain we also obtained positive cultures on the 5th and 7th day, and variable results on the 10th day.

Conditions of culture.—In these experiments we attempted to ascertain the ingredients and concentration necessary for growth. Serum is essential. Guinea-pig and rabbit serum are equally satisfactory. Growth occurs in serum concentrations of 1 : 6 and 1 : 12, but not when the concentration is reduced to 1 : 24.

In place of tunica tissue, any other endothelial membrane, either of the rat, guinea-pig or rabbit may be used. In addition to tunica we used pericardium, mesentery and peritoneum of guinea-pigs with good results; the

* Most of the experiments were made with the rat strain. Consequently, reference to the strain is made only in those experiments in which the louse virus was used.

last was also used by Nigg and Landsteiner. The tunica vaginalis is, however, to be preferred to the others, because the testes can be removed without destroying the animal, and this membrane, having few elastic fibres, can be minced up more easily.

Locke solution can be used in place of Tyrode, but glycosal (*i. e.* Tyrode solution without calcium bicarbonate) is unsatisfactory. In order to ascertain whether the bicarbonate performs a function other than buffer, the required amount of $M/15$ K_2HPO_4 was added to glycosal solution in place of the bicarbonate. The results were equally satisfactory, showing that bicarbonate acts as a reaction regulator. No growth was obtained when broth was used in place of Tyrode solution.

Distribution of Rickettsia *in the animal.*—Direct cultures were made with different endothelial membranes taken from infected animals. Positive cultures were obtained from the mesentery and pericardium of infected rats, as well as from the mesentery of infected guinea-pigs.

Survival of Rickettsia in vitro.—Infected testicles kept 24 hours in a sterile petri dish at room temperature still give positive cultures. An emulsion of *Rickettsia*-infected tissue or culture in saline loses its infectivity for guinea-pigs after 24 hours at 37° C. The duration of life of the cultures is from 15–20 days. After this time subcultures or infection of guinea-pigs no longer give uniform results, even though on staining the *Rickettsia* appear to be still numerous and intact. Nigg (1935) reports a period of viability of her cultures of several months when kept at 37° C., and of less than a month when grown at 20° C. We have not grown our cultures at 37° C., and cannot, therefore, say whether the different results obtained by us are due solely to the temperature, or to other factors, such as difference in the strains.

DISCUSSION.

The results recorded in this paper raise a number of interesting points.

The findings again emphasize the differences between the murine and human strains of *Rickettsia*. The distinctive localization of these strains has already been noted by various observers. The cultivation experiments confirm and extend these observations. The occasional finding of *Rickettsia* in the tunica of guinea-pigs infected by the human strain by some authors and not by others is accounted for by the fact that these organisms regularly infect the mesorchium, where they may at times be found, but not the tunica. Why they should invade the one tissue and not the other is not clear, but this fact further emphasizes the differences in the invasiveness of the rat and human strain.

In vitro the conditions of growth and survival of the two strains appear to be the same. The same tissues, same serum and fluids and same temperature appear to be required by both.

SUMMARY.

A technique is described for the cultivation of *Rickettsia* by using infected tissues for primary cultures. This technique has enabled us to study a number

of problems relating to the growth of these organisms, the time of their appearance and disappearance in the infected animal, etc.

It was found that *Rickettsia* grows in rabbit as well as in guinea-pig serum, and that a minimum serum concentration of 1 : 12 is essential. Tyrode or Locke solutions can be used—the bicarbonate acting as buffer. The favourable reaction is between the neutral point and pH 7·6. Any endothelial membrane will serve as culture tissue.

The murine strain invades the tunica of the guinea-pig on the 4th day, reaches a maximum on the 6th, and disappears between the 10th and 12th day of the infection. The louse-borne (European) strain does not invade the tunica of the guinea-pig, but is found in the mesorchium. It appears on the 5th day, and reaches a maximum on the 7th day after the infection. On this day an occasional culture of the tunica is also positive. The rat strain has also been cultivated from the mesentery, peritoneum and pericardium.

Infected testicles may be kept 24 hours at room temperature without injury to the *Rickettsia*. Cultures incubated at 28–30° C. survive, and are infective up to 15–20 days. Subcultures retain their virulence for guinea-pigs.

REFERENCES.

KLIGLER, I. J., AND ASCHNER, M.—(1933) *Proc. Soc. exp. Biol., N.Y.*, **31**, 349.—(1934) *Ibid.*, **31**, 808.—(1934) *Brit. J. exp. Path.*, **15**, 337.
NIGG, C., AND LANDSTEINER, K.—(1932) *J. exp. Med.*, **55**, 563.
NIGG, C.—(1935) *Ibid.*, **61**, 17.

EXPLANATION OF PHOTOGRAPHS.

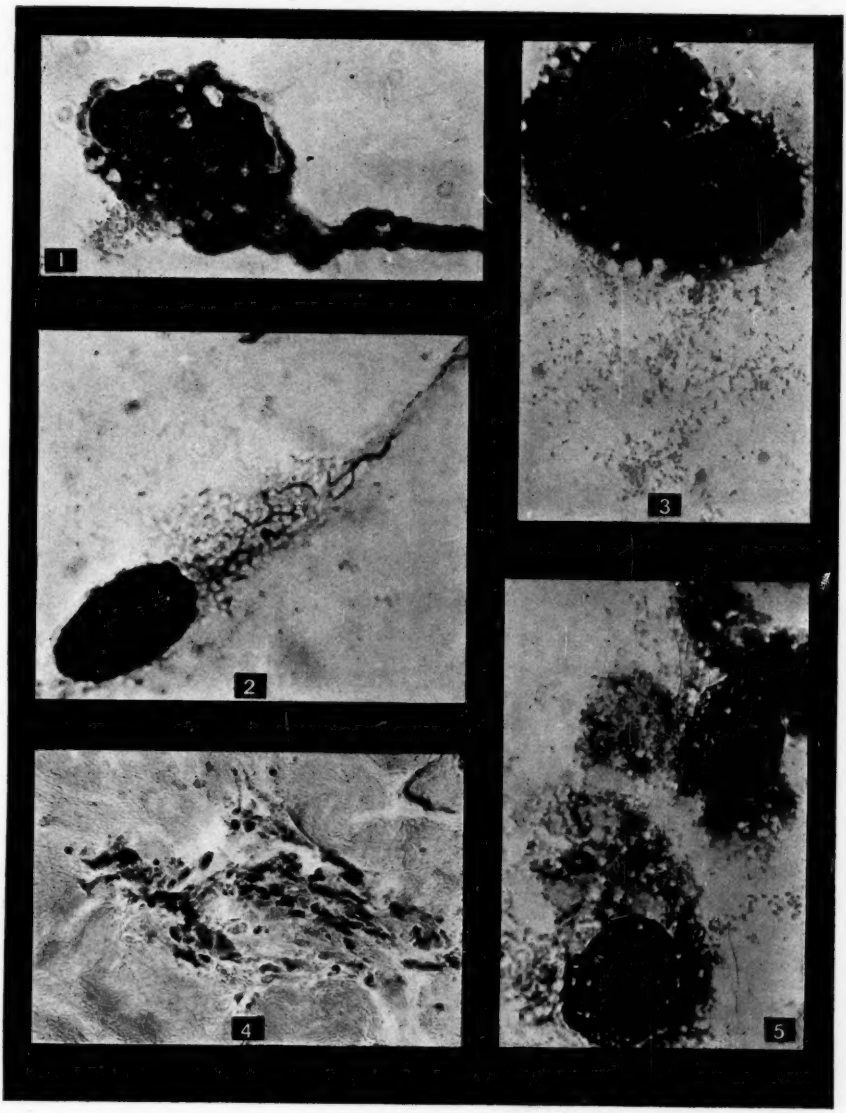
FIG. 1.—Human strain in culture. Stained with Giemsa, × 1000.

FIG. 2.—Murine strain in young culture showing thread forms. Stained with Giemsa. × 1000.

FIG. 3.—A cell from a culture of the murine strain heavily infected with *Rickettsia*, which obscure the cell nucleus. Stained with Giemsa. × 1000.

FIG. 4.—Section through a granule of tissue from a culture. Fixed with Bouin and stained with Giemsa. × 80. The heavily-stained cells are infected with *Rickettsia*.

FIG. 5.—Smear from a murine culture showing the typical appearance of infected tunica cells. Stained with Giemsa. × 1000.



Aschner and Kligler.

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THE PRODUCTION AND PERSISTENCE OF H AND O AGGLUTININS AND COMPLEMENT-FIXING BODIES IN PERSONS INOCULATED WITH TYPHOID ENDOTOXOID VACCINE.

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In previous papers dealing with immunization by "formol endotoxoid" antigen for the preparation of therapeutic antityphoid serum (Grasset and Gory, 1927; Grasset, 1930, 1931), and for prophylactic and therapeutic purposes in human beings (Grasset, 1934, 1935), reference was made to the high degree of immunity conferred on experimental animals to both virulent *B. typhosus* cultures and the specific endotoxin, and to the high H and O agglutinin content of the sera of such immunized animals and human subjects. Results of protection tests have been published in a previous communication (Grasset, 1933), and we shall limit the scope of the present paper to the production of complement-fixing bodies and H and O agglutinins in the sera of immunized subjects. As these observations were commenced before the publications of Felix, Bhatnagar and Pitt (1934, 1935) on the Vi antigen we shall not include observations on this subject.

There is marked divergency in the results obtained by various observers on the production of O agglutinins in the sera of persons inoculated with typhoid vaccine. Some workers find that prophylactic typhoid inoculation produces no small-flaking agglutinins, others that these agglutinins are produced, but only in small quantity, and some that they are stimulated to a degree equal to that found in active typhoid infection.

Felix (1924), referring to a group of persons inoculated with T.A.B. vaccine, states: ". . . not in a single one of these cases is small-flaked agglutination to be found, even in the strongest serum concentration". He employs this finding (1929, 1930) in differentiating agglutinins produced in active infection from those which occur after prophylactic inoculation. Burnet (1924*a*, 1924*b*) arrived at similar conclusions. Stuart and Krikorian (1928) and Pijper (1930) confirmed the results obtained by Felix, though Pijper states that he has "not had much opportunity of studying this side of the agglutination problem" and quotes only five cases. Whitehead (1927), in a series

of seven inoculated cases, detected O agglutinins in only one, and this in a serum dilution not higher than 1 in 50. Downie and Fairbrother (1934) found that " agglutinins for the O antigen were almost invariably absent " from sera of individuals inoculated with T.A.B. vaccine, in the majority of cases between the years 1914–1918.

Gardner (1929) demonstrated O agglutinins in a titre of 1 in 800 in the sera of individuals shortly after inoculation, and " has seen traces at 1 in 1000 soon after the second dose " (1930). Mudd (1932) detected small flaking agglutinins in a serum dilution of 1 in 320 in vaccinated persons ; Smith (1932) in a titre of 1 in 200 ; Wyllie (1932) in a titre of 1 in 250 ; Horgan (1932) up to serum dilutions of 1 in 1250, falling to 1 in 500 after six weeks ; Dulaney, Wikle *et al.* (1933) found the O titre to vary from 1 in 40 to 1 in 320 ; Giglioli (1933) in a titre of 1 in 320. Dennis and Berberian (1934) found the " average titre for O agglutinins " to be 1 in 600. Stuart and Krikorian (1934) showed that a non-phenolized vaccine could stimulate the production of small-flaking agglutinins in serum dilutions of 1 in 2000. Bole (1935), examining a group of 28 vaccinated non-typhoid cases, found that " in no case did the O titre exceed 1 in 80 ".

The inconsistency of these results may be partly accounted for by the variation in the vaccines used and the technique employed in the agglutination tests.

An investigation carried out in 1933–1934 by one of us (E. G.) on approximately 100 non-European Rand Mine labourers showed that O agglutinins were almost invariably produced by typhoid endotoxoid vaccine, and in some cases could be detected in serum dilutions of 1 in 640 two to three weeks after a second inoculation. As this group could not be maintained under constant observation it was decided to inoculate a further group of individuals, who could be kept under constant personal supervision, and on whom serum analysis could be performed at intervals over a considerable period. The non-European members, 55 in number, of this Institute, all adults, were accordingly inoculated wtih typhoid endotoxoid vaccine (Batch No. S.19) prepared for human use at the South African Institute for Medical Research. This vaccine was approximately 30 p.c. more concentrated than that used in 1933. Each person received three injections subcutaneously (0·5 c.c., 1·0 c.c., 1·0 c.c.). Serum analysis was made before inoculation, after each inoculation, and at periods after the last injection.

A formolized broth suspension of *B. typhosus* H.901 was used to detect H agglutinins ; for O agglutinins an alcoholic suspension of *B. typhosus* O.901. These antigens were prepared in bulk and stored at 0°–5° C., tested from time to time to detect any possible variation in agglutinability, and the same batch was used throughout the investigation. Details of the preparation of the antigens and technique of the tests employed are described in previous papers (Lewin, 1934, 1935). H readings were taken after two hours' incubation in a water-bath at 52° C., O readings after a further 22 hours. The readings were made in daylight with the aid of a watchmaker's lens. Any doubtful result was recorded as negative. The agglutination tests were performed in serum dilutions of 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600 and 1 in 3200.

The results obtained are tabulated in Table I. Cases 40, 41 and 48 had been inoculated with a less concentrated endotoxoid vaccine one year previously, and Case 21 has a doubtful history of T.A.B. inoculation in 1916. It will be noticed that the sera of 8 cases, excluding Cases 41 and 21, exhibited agglutinins before the administration of vaccine.

These results show that in the large majority of cases both H and O agglutinins are produced, the former in much higher titre than the latter, and in several cases small-flaking agglutinins can be detected in serum dilutions of at least 1 in 800. In addition, it is seen that O agglutinins produced by typhoid endotoxoid vaccine are detectable in the greater number of cases in serum dilutions of at least 1 in 200—the accepted " diagnostic titre " for local cases ill with symptoms of typhoid fever. An agglutination test in this one dilution

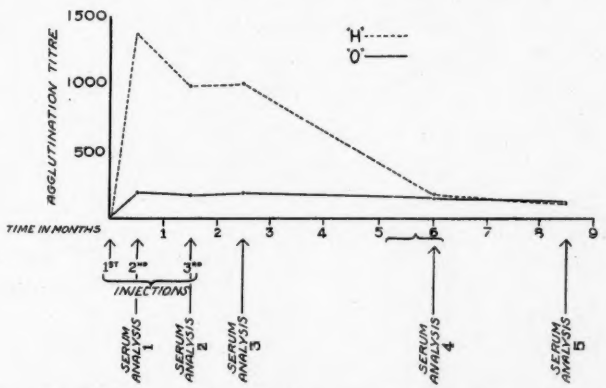

FIG. 1.—Graph showing average H and O agglutination titres after typhoid endotoxoid inoculation.

of the serum of a previously inoculated individual sick with typhoidal symptoms will thus not aid the diagnosis. This finding is contradictory to conclusions drawn by Felix (1924, 1929, 1930), Burnet (1924*a*, 1924*b*), and Pijper (1930). This fact must also be considered in the routine investigation for the detection of typhoid carriers by means of H and O agglutination, especially in an inoculated population such as the Rand non-European mine labourers, of whom over one hundred thousand have, up to date, received typhoid endotoxoid vaccine.

The variation in the H and O agglutinin titre after each injection and at intervals after the third are best illustrated by means of a graph (Fig. 1), where the average agglutinin titres are plotted as ordinates, and the time intervals after injection as abscissæ. The O curve, while it does not rise as much as the H, maintains its height more uniformly. It should be noted that, owing to the serum dilutions in which the tests were performed, the titres here recorded are not the highest obtainable.

In order to determine the effect which would be produced by concentrating

TABLE I.—*H and O Agglutination and Typhoid Complement Fixati*

Case.	Previous inoculation or infection.	Agglutinin titre previous to inoculation.		Interval between first injection and serum analysis 1.	Serum analysis 1 (after first injection).				Interval between second injection and serum analysis 2.	Serum analysis 2 (after second injection).				
					Agglutinin titre.		T.C.F.			Agglutinin titre.		T.C.F.		
		H.	O.		H.	O.	M.H.D. 3.	5. 7.		H.	O.	M.H.D. 3.	5.	7.
1	*Nil*	0	0	2 weeks	400	100	+	— —	4 weeks	200	0	+	—	—
2	,,	0	0	,,	3,200	0	—	— —	,,	800	100	—	—	—
3	,,	0	0	,,	3,200	100	—	— —	,,	800	100	—	—	—
4	,,	0	0	,,	800	100	—	— —	,,	800	100	—	—	—
5	,,	0	100	,,	1,600	200	—	— —	,,	1,600	200	—	—	—
6	,,	100	400	,,	3,200	800	—	— —	,,	3,200	400	—	—	—
7	,,	0	0	,,	800	0	—	— —	,,	400	0	—	—	—
8	,,	0	0	,,	800	200	—	— —	,,	800	200	—	—	—
9	,,	0	0	,,	3,200	400	+	± ±	,,	3,200	200	—	—	—
10	,,	0	0	,,	200	100	—	— —	,,	1,600	200	—	—	—
11	,,	0	0	,,	3,200	800	—	— —	,,	3,200	800	+	+	+
12	,,	0	0	,,	1,600	100	—	— —	,,	800	100	—	—	—
13	,,	0	0	,,	800	100	—	— —	,,	400	100	—	—	—
14	,,	0	100	,,	1,600	800	+	— —	,,	1,600	200	—	—	—
15	,,	0	0	,,	800	100	—	— —	,,	400	100	—	—	—
16	,,	0	0	,,	800	100	—	— —	,,	400	0	—	—	—
17	,,	0	0	,,	1,600	200	—	— —	,,	800	100	+	+	+
18	,,	0	0	,,	800	400	+	— —	,,	800	200	+	+	+
19	,,	0	0	,,	3,200	800	+	+ +	,,	3,200	800	+	+	+
20	,,	0	100	,,	800	100	—	— —	,,	200	100	—	—	—
21	T.A.B. 1916 ?	0	100	,,	3,200	400	—	— —	,,	1,600	400	—	—	—
22	*Nil*	0	0	,,	100	0	—	— —	,,	200	0	—	—	—
23	,,	0	0	,,	100	0	—	— —	,,	200	200	+	+	+
24	,,	0	0	,,	3,200	0	—	— —	,,	1,600	0	—	—	—
25	,,	0	0	,,	3,200	200	—	— —	,,	1,600	0	—	—	—
26	,,	0	0	,,	800	400	—	— —	,,	200	200	—	—	—
27	,,	0	0	,,	400	200	—	— —	,,	400	100	—	—	—
28	,,	0	0	,,	3,200	100	—	— —	,,	800	0	—	—	—
29	,,	0	0	,,	0	0	—	— —	,,	400	100	Anti-comp.		
30	,,	0	100	,,	400	200	—	— —	,,	200	200	—	—	—
31	,,	0	0	,,	1,600	0	—	— —	,,	800	0	±	—	—
32	,,	0	0	,,	3,200	100	—	— —	,,	3,200	100	—	—	—
33	,,	0	0	,,	800	100	—	— —	,,	400	100	—	—	—
34	,,	0	100	,,	1,600	100	+	— —	,,	1,600	100	+	+	+
35	,,	400	200	,,	800	200	—	— —	,,	400	100	+	±	—
36	,,	0	0	,,	800	0	—	— —	,,	400	0	—	—	—
37	,,	0	0	,,	3,200	100	—	— —	,,	1,600	100	—	—	—
38	,,	0	200	,,	3,200	400	—	— —	,,	3,200	800	—	—	—
39	,,	0	0	,,	400	200	—	— —	,,	200	200	—	—	—
40	T. endotoxoid, March, 1934	0	0	,,	800	0	—	— —	,,	400	200	—	—	—
41	Ditto	0	100	,,	200	200	—	— —	,,	100	200	—	—	—
42	*Nil*	0	0	,,	200	0	—	— —	..	..	..	..		
43	,,	0	0	,,	800	0	—	— —	..	..	..	..		
44	,,	0	0	4 weeks	800	0	—	— —	1 week	200	100	—	—	—
45	,,	0	0	,,	200	0	—	— —	,,	200	0	—	—	—
46	,,	0	0	1 week	100	200	—	— —	2½ weeks	400	200	—	—	—
47	,,	0	0	,,	0	100	—	— —	,,	200	100	—	—	—
48	T. endotoxoid, March, 1934	0	0	2 weeks	400	400	—	— —	2 weeks	400	800	—	—	—
49	*Nil*	0	0	,,	3,200	400	+	— —	3 weeks	1,600	400	+	—	—
50	,,	0	0	,,	800	200	±	— —	,,	200	200	±	—	—
51	,,	0	0	,,	800	200	—	— —	,,	400	200	—	—	—
52	,,	0	0	,,	400	200	+	— —	,,	200	200	+	—	—
53	,,	0	0	,,	400	400	—	— —	,,	1,600	100	—	—	—
54	,,	0	0	,,	400	100	—	— —	,,	200	200	—	—	—
55	,,	0	0	,,	3,200	400	—	— —	,,	1,600	400	—	—	—

Fixation Tests after Typhoid Endotoxoid Vaccine Inoculation.

Interval between third injection and serum analysis 3.	Serum analysis 3 (after third injection).				Interval between third injection and serum analysis 4.	Serum analysis 4.					Interval between third injection and serum analysis 5.	Serum analysis 5.				
	Agglutinin titre.		T.C.F.			Agglutinin titre.		T.C.F.				Agglutinin titre.		T.C.F.		
	H.	O.	M.H.D. 3.	5.	7.		H.	O.	M.H.D. 3.	5.	7.		H.	O.	M.H.D. 3.	5.	7.
..	..	..	..			6½ months	100	0	—	—	—	7½ months	100	0	—	—	—
4 weeks	400	200	—	—	—	4¾ months	0	100	—	—	—	7 months	0	100	—	—	—
,,	800	100	—	—	—	,,	200	100	—	—	—	,,	200	100	—	—	—
,,	800	100	—	—	—	,,	100	100	—	—	—	,,	100	100	—	—	—
,,	3,200	400	—	—	—	,,	0	400	—	—	—	,,	0	400	—	—	—
,,	1,600	800	—	—	—	,,	400	800	—	—	—	,,	400	800	—	—	—
,,	400	0	—	—	—	,,	0	0	—	—	—	,,	0	0	—	—	—
,,	800	400	±	—	—	4 months	200	400	±	—	—	..	..	..	..		
,,	3,200	100	±	—	—	4¾ months	400	100	—	—	—	7 months	200	0	—	—	—
,,	1,600	200	—	—	—	,,	200	200	—	—	—	..	..	..	..		
,,	1,600	800	+	+	+	,,	400	800	+	—	—	7 months	200	400	—	—	—
,,	1,600	100	—	—	—	,,	200	100	—	—	—	,,	0	100	—	—	—
,,	800	100	+	—	—	,,	0	100	—	—	—	,,	0	100	—	—	—
,,	1,600	200	±	—	—	,,	100	200	+	—	—	,,	100	200	—	—	—
,,	800	100	—	—	—	..	..	..	..			..	..	..	..		
,,	3,200	100	—	—	—	4¾ months	100	100	—	—	—	7 months	100	0	—	—	—
,,	1,600	100	+	+	+	,,	800	0	+	+	—	,,	400	0	±	—	—
,,	800	200	+	+	+	,,	400	100	+	+	±	,,	200	100	+	±	—
..	..	..	..			..	400	200	+	+	+	,,	100	200	—	—	—
,,	400	200	—	—	—	4¾ months	0	200	—	—	—	,,	0	200	—	—	—
,,	3,200	800	—	—	—	2 months	800	800	—	—	—	..	..	..	..		
,,	400	0	—	—	—	,,	100	100	—	—	—	..	..	..	..		
,,	800	100	+	+	+	4¾ months	100	100	+	±	—	7 months	100	100	+	—	—
,,	1,600	100	—	—	—	,,	400	0	—	—	—	,,	400	0	—	—	—
,,	1,600	100	—	—	—	2 months	400	100	—	—	—	..	..	..	..		
,,	200	200	—	—	—	4¾ months	0	200	—	—	—	..	..	..	..		
,,	800	100	—	—	—	,,	100	100	—	—	—	..	..	..	..		
,,	400	100	—	—	—	,,	100	100	—	—	—	7 months	100	100	—	—	—
,,	800	100	Anti-comp.			,,	0	0	—	—	—	,,	0	0	—	—	—
,,	100	200	—	—	—	3 months	0	200	—	—	—	..	..	..	..		
,,	800	0	±	—	—	4¾ months	400	0	±	—	—	7 months	200	0	—	—	—
,,	1,600	100	±	—	—	,,	400	100	—	—	—	,,	400	100	—	—	—
,,	400	100	—	—	—	4 months	200	100	—	—	—	..	..	..	..		
,,	1,600	100	—	—	—	4¾ months	100	100	±	—	—	7 months	100	100	—	—	—
,,	800	100	+	+	+	,,	0	100	±	—	—	,,	0	100	±	—	—
,,	400	0	—	—	—	2 months	100	0	—	—	—	..	..	..	..		
,,	800	100	—	—	—	4¾ months	400	100	—	—	—	7 months	200	100	—	—	—
,,	3,200	800	—	—	—	,,	400	400	—	—	—	,,	400	400	—	—	—
,,	200	100	—	—	—	3 months	200	0	—	—	—	..	..	..	..		
,,	400	200	+	±	—	4¾ months	100	0	—	—	—	7 months	0	0	—	—	—
,,	0	200	—	—	—	,,	0	200	—	—	—	,,	0	200	—	—	—
..	..	..	..			..	..	..	..			..	..	..	..		
..	..	..	..			..	..	..	..			..	..	..	..		
3 weeks	200	200	—	—	—	4 months	200	100	—	—	—	6½ months	0	100	—	—	—
,,	200	100	—	—	—	,,	0	100	—	—	—	,,	0	0	—	—	—
2 weeks	400	200	—	—	—	3¾ months	200	0	—	—	—	6 months	0	0	—	—	—
,,	800	200	—	—	—	,,	0	0	—	—	—	..	..	..	..		
3 weeks	400	800	—	—	—	,,	100	400	—	—	—	6 months	100	400	—	—	—
2 weeks	800	400	+	±	—	..	..	..	..			..	..	..	..		
,,	200	200	—	—	—	..	..	..	..			..	..	..	..		
,,	400	100	—	—	—	..	..	..	..			..	..	..	..		
,,	200	200	+	—	—	..	..	..	..			..	..	..	..		
3 weeks	800	100	—	—	—	..	..	..	..			..	..	..	..		
,,	200	200	—	—	—	..	..	..	..			..	..	..	..		
,,	400	400	—	—	—	..	..	..	..			..	..	..	..		

TABLE II.—*H and O Agglutination and Complement-Fixation Tests after Inoculation with Concentrated Typhoid Endotoxoid.*

Case.	Agglutination and T.C.F. tests previous to inoculation.					Serum analysis 10 days after first inoculation (0·5 c.c.).					Serum analysis 4 days after second inoculation (1·0 c.c.).					Serum analysis 10 days after second inoculation.				
	Agglutination titre.		T.C.F. M.H.D.			Agglutination titre.		T.C.F. M.H.D.			Agglutination titre.		T.C.F. M.H.D.			Agglutination titre.		T.C.F. M.H.D.		
	H.	O.	3.	5.	7.	H.	O.	3.	5.	7.	H.	O.	3.	5.	7.	H.	O.	3.	5.	7.
1	0	0	—	—	—	10,240	1,280	±	—	—	5,120	640	±	—	—	1,280	320	±	—	—
2	0	0	—	—	—	10,240	5,120	+	±	—	2,560	5,120	+	+	+	2,560	5,120	+	±	—
3	0	80	—	—	—	160	640	±	—	—	..	..	.	..	.	..	..	.	..	
4	0	0	—	—	—	2,560	80	+	—	—	1,280	160	+	+	±	2,560	80	—	—	—
5	0	0	—	—	—	1,280	640	+	±	—	5,120	1,280	±	—	—	10,240	640	—	—	—
6	0	0	—	—	—	5,120	160	+	±	—	10,240	160	±	—	—	10,240	640	±	—	—
7	0	0	—	—	—	640	640	+	±	—	640	640	±	—	—	640	640	±	—	—

the antigen used for the inoculations, 7 non-European adults were inoculated with typhoid endotoxoid vaccine of a concentration 2·25 times that used on the 55 persons detailed above. The agglutinin and complement-fixing body response is tabulated in Table II.

It will be noticed that in one case this concentrated vaccine produced O agglutinins in a serum dilution of 1 in 5120, and in another two cases the O titre was 1 in 1280. All cases, in the first serum analysis, gave a positive complement-fixation reaction.

Mice and rabbits were inoculated with endotoxoid vaccine of similar concentration to that used in the group of 55 humans. In mice O agglutinins were detected in a serum dilution of 1 in 80 ten days after subcutaneous inoculation with 0·5 c.c. This titre was increased to 1 in 320 after two inoculations of 1·0 c.c. Rabbits, ten days after one subcutaneous injection of 0·5 to 1·0 c.c., developed O agglutinins in serum dilutions varying from 1 in 2560 to 1 in 5120. Before inoculation the sera of these animals did not reveal the presence of agglutinins in a dilution of 1 in 20.

Table I shows that, at some stage during the course of inoculation, the sera of 18 of the 55 cases (approximately 32 p.c.) gave a positive typhoid complement-fixation reaction ; in 9 instances this occurred after the first injection. Felke (1915) found the complement-fixation reaction to be constantly negative in a group of 39 persons vaccinated against typhoid. Garbat (1916) found that the sera of individuals who received two injections rarely gave a positive reaction, whereas after three injections the reaction was positive in 50 p.c. of cases. Pijper (1923) found the test positive in 5 out of 40 cases inoculated with T.A.B. vaccine. Of these positive cases he states, " . . . a history of having had typhoid fever was given by three. A similar history might therefore be assumed in the remaining two cases ".

SUMMARY.

(1) The sera of individuals inoculated with typhoid endotoxoid vaccine produced by the South African Institute for Medical Research showed the presence of H and O agglutinins, in the large majority of cases in high titre. A concentrated antigen produced a higher O agglutinin response than this regular product.

(2) The titre for H agglutinins shortly after inoculation is much higher than for O, but the titre of the latter remains at a more constant level, the H titre falling rapidly.

(3) At some stage during inoculation complement-fixing bodies were present in approximately 32 p.c. of individuals inoculated with endotoxoid vaccine of a concentration as issued for human use. A more concentrated antigen, in a series of 7 individuals, produced a positive complement-fixation reaction in all cases.

(4) Of sera from 33 cases examined seven months after the last injection, 4, or 12 p.c., gave a positive complement-fixation reaction.

(5) An O agglutination test performed in a serum dilution of 1 in 200 will not aid the diagnosis in the case of a person with a febrile illness who has been recently inoculated with typhoid endotoxoid vaccine. Inoculation of a

population with such a vaccine will limit the use of H and O agglutination tests and the typhoid complement-fixation test for the detection of the carrier state.

REFERENCES.

Bole, R.—(1925) *J. Lab. Clin. Med.*, **20**, 638.

Burnet, F. M.—(1924*a*) *Med. J. Australia*, **1**, 205.—(1924*b*) *Brit. J. exp. Path.*, **5**, 251.

Dennis, E. W., and Berberian, D. A.—(1934) *Amer. J. Hyg.*, **20**, 469.

Downie, A. W., and Fairbrother, R. W.—(1934) *Brit. med. J.*, **1**, 55.

Dulaney, A. D., *et al.*—(1933) *J. Immunol.*, **24**, 229.

Dulaney, A. D., and Wikle, W. T.—(1933) *Ibid.*, **24**, 235.

Felix, A.—(1924) *J. Immunol.*, **9**, 115.—(1929) *J. Hyg.*, Camb., **28**, 418.—(1930) *Lancet*, **1**, 505.

Felke.—(1915) *München. med. Wschr.*, **62** (abst. *J. Amer. Med. Ass.*, 1915, **64**, 2028).

Garbat, A. L.—(1916) *J. Immunol.*, **1**, 391.

Gardner, A. D.—(1929) *J. Hyg.*, Camb., **28**, 376.—(1930) *Lancet*, **1**, 427 (correspondence).

Giglioli, G.—(1933) *J. Hyg.*, Camb., **33**, 387.

Grasset, E.—(1930) *J. med. Ass. S. Afr.*, **4**, 380.—(1931) *C. R. Soc. Biol.*, Paris, **106**, 810.—(1933) *Ibid.*, **113**, 20.—(1934) *Ibid.*, **115**, 1485, 1599.—(1935) *J. med. Ass. S. Afr.*, **9**, 208.

Idem and Gory, M.—(1927) *C. R. Soc. Biol.*, Paris, **96**, 180, 1211.

Horgan, E. S.—(1932) *J. Hyg.*, Camb., **32**, 523.

Lewin, W.—(1934) *J. med. Ass. S. Afr.*, **8**, 731.—(1936) *Ibid.*, **10**, 45.

Mudd, S.—(1932) *J. Immunol.*, **23**, 81.

Pijper, A.—(1923) *S. Afr. med. Record*, **21**, 51.—(1930) *J. Hyg.*, Camb., **29**, 380.

Smith, J.—(1932) *Ibid.*, **32**, 143.

Stuart, G., and Krikorian, K. S.—(1928) *Ibid.*, **27**, 105.—(1934) *Lancet*, **2**, 644.

Whitehead, N. T.—(1927) *J. R. Army med. C.*, **49**, 241.

Wyllie, J.—(1932) *J. Hyg.*, Camb., **32**, 375.

PURIFIED FOOT-AND-MOUTH DISEASE VIRUS. I: STUDIES ON SOME OF ITS PHYSICAL PROPERTIES.*

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IT is becoming increasingly evident that the next stage in the study of viruses must be the investigation of the antigenic and physical-chemical properties of purified preparations of these infective agents. Attempts so far made to purify viruses have been based for the most part on their surface properties, which in general are very similar to those of other substances in a state of colloidal dispersion—*e. g.* reversible adsorption on to surfaces of opposite electric charge, and migration in an electric field. However, owing to the fact that the tissue and serum proteins, with which viruses are always associated, behave in adsorption and electrical migration experiments very similarly to the virus particles themselves, separation by these methods, when at all possible, is extremely difficult, and the final product invariably shows a considerable drop in virus potency.

Recently Janssen (1936) has claimed that highly potent preparations of pure foot-and-mouth disease virus may be obtained by adsorbing the virus on to calcium sulphate, precipitated by the addition of 33 p.c. of an alcohol-ether (70 : 30) mixture to a suspension of virulent fluid in a saturated gypsum solution while maintaining the temperature throughout at – 10° C. The precipitate, which contains most of the virus and some tissue protein, may be separated by centrifugation and resuspended in a saturated gypsum solution. The complete removal of the extraneous protein matter can be effected by a repetition of the process. The final dissolution of the precipitate, with the adsorbed virus, may be made in water instead of gypsum solution. The important features of this technique appear to be (1) working at extremely low temperatures, and (2) carrying out the manipulations as quickly as possible in order to minimize any deleterious action of the alcohol on the virus.

Knowledge of the sizes of the different viruses has enabled alternative methods to be devised, viz. washing processes based on differential centrifugation and filtration. Thus by means of fractional centrifugation it has been possible with some of the largest viruses such as vaccinia (Craigie, 1932; Parker and Rivers, 1935), and psittacosis (Bedson, 1932), to spin down the virus elements, and after discarding the supernatant fluid, to resuspend the sediment in a chosen medium. Repetition of this process leads to a final product in which the virus has been largely freed from extraneous protein with little loss in potency. The method has been applied also to one of the larger bacteriophages (Schlesinger, 1933). It cannot at present be used

* This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given their permission for the publication of this paper.

successfully for viruses smaller than 100 mμ. On the other hand, when the washing is carried out on a suitable membrane the method becomes of much wider application and can embrace all the viruses, and has recently been used with success in purifying enzymes (Dubos and Bauer, 1935).

Galloway and Elford (1931) showed that gradocol membranes of average pore diameter (A.P.D.) 25 mμ. or less completely retained the virus of foot-and-mouth disease, while serum proteins are not held back entirely until porosities smaller than 10 mμ. are employed. Thus the possibility is afforded of washing foot-and-mouth disease virus on a membrane of 25 mμ. A.P.D. by a procedure similar to that outlined for ectromelia virus by Barnard and Elford (1931). The method has also been applied with more or less success to obtain purified preparations of Rous sarcoma virus (Elford and Andrewes, 1935) and bacteriophages (Elford and Andrewes, 1932). However, each virus or bacteriophage presents its own problem in purification, particularly in relation to stability under different conditions of pH and medium. This fact has been well illustrated in our studies with foot-and-mouth disease virus, and may serve to explain certain difficulties that have arisen in applying the method to some other viruses.

EXPERIMENTAL METHODS.

Stock filtrates.—Guinea-pig vesicle lymph was diluted 1 : 20 in phosphate buffer solution (Sörensen) at pH 7·6. The phosphate concentration usually employed was *M*/45, but *M*/30 and *M*/90 solutions have been found to serve equally well. A bacteriologically sterile stock filtrate was then prepared as in our previous studies, preliminary clarification by centrifugation, and sand and pulp filtration, being followed by filtration through a gradocol membrane of A.P.D. 0·6 μ. Such filtrates generally proved infective in a 1/100,000 dilution.

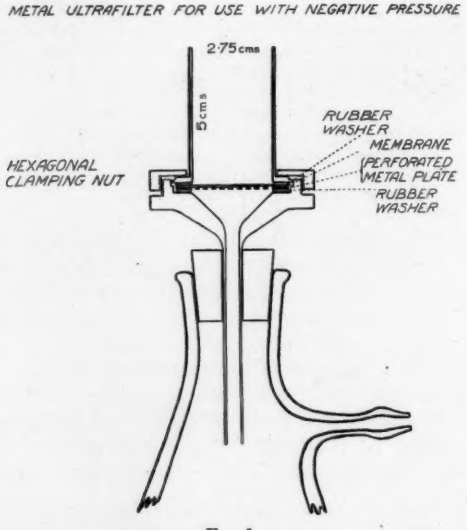

FIG 1.

Purification of virus.—The grade of membrane employed has been of A.P.D. 25 mμ., *i. e.* the highest porosity consistent with complete retention of foot-and-mouth disease virus. For purification by the washing technique it has been found most convenient to employ a type of ultrafilter for use with negative pressures, as shown in Fig. 1. The method of mounting and clamping the membrane is seen to be the same as in the ultrafilter for positive pressures previously designed (Barnard and Elford, 1931). However, the open top to the negative pressure filter facilitates the observation of the progress of filtration, and also, of course, fresh additions of the washing medium may be made without interrupting the suction.

The general procedure has been to charge the filter (of the dimensions indicated) with 20 c.c. of the stock filtrate. Then, with the best vacuum obtainable with the ordinary form of glass water-pump, filtration was allowed to proceed until the volume of liquid above the membrane was reduced to about 1 c.c. This was then diluted to the original volume with the washing medium—usually *M*/45 phosphate buffer solution at pH 7·6. The process of filtration followed by dilution was repeated four times, and for the whole process two days were required. (*N.B.*—The filter was placed in cold storage at 2° C. overnight.) This sufficed to provide a virus suspension which, while it proved to be fully potent, gave no indication of protein by the sulphosalicylic acid test. The stock virus filtrates, as also the primary filtrate through a 25mμ. membrane, invariably gave a positive protein test when diluted 1 : 100, but were negative in a 1 : 1000 dilution. It is clear therefore that by the washing treatment the virus was freed from extraneous protein to the extent of upwards of 99 p. c.

POTENCY OF PURIFIED VIRUS PREPARATIONS.

Up to the present twelve different preparations of purified virus have been made. Eleven of these were made from the G.F. strain of virus (Vallée + Carrée " O " type), and one from the Riems C strain of virus (Waldmann and Trautwein " C " type). Titrations of the virus were made by inoculation of the different dilutions in series intradermally into the metatarsal pads of each of two guinea-pigs. The titrations of purified virus preparations, together with those of the corresponding stock filtrates, are given in Table I. Nine of the twelve preparations of purified virus produced a generalized infection in guinea-pigs in a dilution of 1 : 100,000 (equivalent to a 1 : 2,000,000 dilution of vesicle lymph), and three in a dilution of 1 : 10,000. These titres compared very favourably with those of the stock filtrates from which they were prepared. In the only instances where the purified virus showed a lower titre than the unwashed virus (not recorded in the table), the membrane had been allowed inadvertently to become dry during the experiment. It is essential to avoid this to ensure a fully active final product.

STABILITY OF PURIFIED VIRUS PREPARATIONS.

Stability of purified virus compared with that of unpurified virus in* M/45 *phosphate solution buffered at pH* 7·6.—Different samples of purified virus and unpurified virus were kept under the same conditions at 22° C. for periods of 3 and 8 days, and at 0° C. for periods varying from 5–126 days. Titrations of the virus were made in the usual way at the end of the time intervals, and were compared with those obtaining at the commencement of each experiment. The results of these tests are given in Table II. It will be seen that in experiments made at 22° C. no appreciable drop in titre of one preparation of purified

* When speaking of unpurified virus, the stock membrane filtrate of a suspension of vesicle fluid in buffered phosphate solution is always intended unless otherwise stated.

TABLE I.—*Titrations of Purified Preparations of Virus Compared with those of Stock Filtrates from which they were Prepared.*

Date of preparation.	Concentration of buffered phosphate solution.	Purified virus. Dilutions. 1 : 1000.	1 : 10,000.	1 : 100,000.	Stock filtrate (unpurified virus). Dilutions. 1 : 1000.	1 : 10,000.	1 : 100,000.
25.ix.35	*M*/45	++	+0	+0	++	+0	00
2.x.35	*M*/45	++	++	+0	++	++	+0
2.x.35	*M*/45	++	+0	+0	—	—	—
2.x.35	*M*/90	++	+0	+0	++	++	00
2.x.35	*M*/90	++	++	+0	—	—	—
10.x.35	*M*/45	++	++	+0	++	++	+0
31.x.35	*M*/45	++	++	++	++	+0	00
3.xii.35	*M*/45	++	++	+0	++	++	+0
6.i.36	*M*/30	++	++	+0	++	++	+0
17.i.36	*M*/30	++	+0	00	++	+0	00
17.i.36*	*M*/30	++	+0	00	++	+0	00
7.ii.36	*M*/45	++	++	00	++	++	00

Remarks.

pH of buffer solution = 7·6.

* This preparation was made from a Type C strain of virus. All the others were made from a Type 0 strain of virus.

++ = Both guinea-pigs inoculated reacted.

+0 = One out of two guinea-pigs inoculated reacted.

00 = Neither of two guinea-pigs inoculated reacted.

TABLE II.—*Stability of Purified Virus Compared with that of Unpurified Virus in* M/45 *Buffered Phosphate Solution at pH* 7·6.

Time interval and temperature.	Titre.	Purified virus. Dilutions. Undil.	1 : 100.	1 : 1000.	1 : 10,000.	1 : 100,000.
3 days, 22° C.	Original	—	++	++	+0	+0
	Final	—	++	++	++	00
8 days, 22° C.	Original	—	++	++	++	+0
	Final	—	++	00	00	00
5 days, 0° C.	Original	—	++	++	+0	+0
	Final	—	++	++	00	00
6 days, 0° C.	Original	—	++	++	++	+0
	Final	—	++	++	++	+0
8 days, 0° C.	Original	—	++	++	++	+0
	Final	—	++	++	00	00
20 days, 0° C.	Original	—	++	++	++	00
	Final	—	++	++	++	—
36 days, 0° C.	Original	—	++	++	++	+0
	Final	—	++	++	++	—
97 days, 0° C.	Original	—	++	++	++	++
	Final	++	+0	00	00	00

Time interval and temperature	Titre.	Unpurified virus (stock filtrates). Dilutions. 1 : 100.	1 : 1000.	1 : 10,000.	1 : 100,000.
3 days, 22° C.	Original	++	++	++	+0
	Final	++	++	++	00
8 days, 22° C.	Original	++	++	++	+0
	Final	++	++	00	00
..	Original	—	—	—	—
	Final	—	—	—	—
..	Original	—	—	—	—
	Final	—	—	—	—
8 days, 0° C.	Original	++	++	++	+0
	Final	++	++	+0	00
22 days, 0° C.	Original	++	++	++	+0
	Final	++	++	++	—
36 days, 0° C.	Original	++	++	++	+0
	Final	++	++	++	—
126 days, 0° C.	Original	++	++	++	+0
	Final	++	++	00	00

— = No test made.

++ = Both guinea-pigs inoculated showed lesions.

+0 = One of two guinea-pigs inoculated showed lesions.

00 = Neither of two guinea-pigs inoculated showed lesions.

virus and another of unpurified virus had occurred after 3 days. In another experiment after 8 days, however, the titre of the purified virus had fallen from 10^{-5} to 10^{-2}, whereas a sample of unpurified virus kept under the same conditions had fallen only from 10^{-5} to 10^{-3}.

At 0° C. with three different preparations of purified virus kept for periods of 6 days, 20 days and 36 days respectively, there was little or no loss of potency. Two other samples, kept for 5 and 8 days respectively, fell from 10^{-5} to 10^{-3}. Very similar results were obtained with three samples of unpurified virus 8 days, 22 days and 36 days respectively at 0° C. In another experiment purified virus kept for an even longer period at 0° C., viz. 97 days, had fallen off in titre from 10^{-5} to 10^{-2}, whereas in the case of a sample of unpurified virus kept under the same conditions for a period of 126 days, the drop in titre was only from 10^{-5} to 10^{-3}.

The variations in stability amongst different batches of purified virus are probably due to varying degrees of " purity " attained in different experiments. It suggests that the stability of the virus is affected by slight variations in the amount of associated proteins which passes undetected by the sulphosalicylic acid test.

Stability of purified virus in broth compared with its stability in phosphate solution buffered M/45 *at pH* 7·6.—During the course of some experiments on centrifugation to be described in a later paper, evidence was obtained indicating that purified foot-and-mouth disease virus was less stable in ordinary stock digest broth than in buffered phosphate solution. Thus purified virus suspended in phosphate solution buffered *M*/45 at pH 7·6 was diluted (*a*) 1 : 10 in broth, and (*b*) 1 : 10 in the buffered phosphate solution. After standing at 22° C. for about 6 hours, the liquids were titred for potency, with the results given in Table III—Experiment 1. Whereas the virus diluted in the buffered phosphate solution had a titre of about 1 : 2500, the virus diluted in broth had a titre of only about 1 : 100. Further titrations were made when the liquid had been kept 24 hours at 22° C. (*v.* Table III, Experiment 2). The virus suspended in buffered phosphate solution had a titre of about 1 : 250, whereas the virus suspended in broth produced no reactions in guinea-pigs in a dilution of 1 : 10.

A further experiment was made to confirm this observation. Purified virus was diluted (*a*) in broth, (*b*) in buffered phosphate solution, and (*c*) in the primary ultrafiltrate through the 25 mμ. membrane obtained during the washing of the virus. In order to determine whether the results of titrations were affected by the fluid employed for making the dilutions, different liquids were employed (*v.* Table III, Experiment 3). The results confirmed the original observation made in Experiments 1 and 2, that purified virus is not stable in stock digest broth, but is stable in buffered phosphate solution or 25 mμ. ultrafiltrate. There was also some suggestive evidence that the use of broth in making dilutions in potency tests may affect the results adversely.

Relative stability of purified virus as compared with that of unpurified virus in broth, peptone water and M/45 *phosphate buffer.*—As already pointed out above, in all filtration experiments with unpurified virus which have been made (Galloway and Elford, 1931), it has been customary to suspend the virus in broth at pH 7·6, as it filters much more easily in this medium than in phosphate solution buffered at pH 7·6. For many investigations also broth filtrates of

— = No test made. ++ = Both guinea-pigs inoculated showed lesions. +0 = One of two guinea-pigs inoculated showed lesions. 00 = Neither of two guinea-pigs inoculated showed lesions.

TABLE III.—*Stability of Purified Virus in Broth Compared with its Stability in* M/45 *Buffered Phosphate Solution.*

Exp. No.	Medium.	Time interval.	Titration diluting fluid.	Dilutions. Undil.	1 : 10.	1 : 50.	1 : 100.	1 : 250.	1 : 500.	1 : 1000.	1 : 1250.	1 : 2500.
1	Stock digest broth (horse flesh)	6 hours	*M*/45 buffered phosphate solution	—	—	—	+0	—	00	—	—	00
	M/45 buffered phosphate solution	6 „	*M*/45 buffered phosphate solution	—	—	—	++	—	++	—	—	+0
2	Stock digest broth (horse flesh)	24 „	*M*/45 buffered phosphate solution	—	00	00	—	00	—	—	00	—
	M/45 buffered phosphate solution	24 „	*M*/45 buffered phosphate solution	—	++	++	—	+0	—	—	00	—
3	Stock digest broth (horse flesh)	5½ „	Broth	++	—	—	00	—	—	00	—	—
			Ultrafiltrate (A.P.D. 25 mμ.)	—	00	—	00	—	—	00	—	—
	M/45 buffered phosphate solution	5½ „	*M*/45 buffered phosphate solution	++	—	—	++	—	—	+0	—	—
			Broth	++	—	—	+0	—	—	+0	—	—
	Ultrafiltrate (A.P.D. 25 mμ.)	5½ „	*M*/45 buffered phosphate solution	++	—	—	++	—	—	+0	—	—
			Broth	++	—	—	+0	—	—	00	—	—

Temperature = 22° C.
Dilution of virus in different media = 1 : 10.
pH of media = 7·6.

++ = Both guinea-pigs inoculated showed lesions.
+0 = One guinea-pig out of inoculated two showed lesions.
00 = Neither of two guinea-pigs inoculated showed lesions.

TABLE VII.—*Photodynamic Action of Methylene-blue on Purified Virus and Unpurified Virus.*

Experiment No.	Time of irradiation. 1 min.	2 min.	3 min.	4 min.	5 min.	10 min.	15 min.	30 min.	45 min.	Control in dark. 45 min.	Control irradiated without M.B. 45 min.
	PURIFIED VIRUS.										
1	—	—	—	—	00	00	00	00	00	++	++
2	++	++	++	++	+0	00	—	—	—	++	++
	UNPURIFIED VIRUS.										
3	—	—	—	—	++	++	++	++	00	++	++

Remarks.

Concentration of methylene-blue (M.B.) = 1 : 50,000.
++ = Both guinea-pigs inoculated showed lesions.
+0 = One of two guinea-pigs inoculated showed lesions.
00 = Neither of two guinea-pigs inoculated showed lesions.
— = No test made.

unpurified virus have been employed, and these have been of consistently high titre and good stability. Broth filtrates of unpurified virus have been kept at 0° C. for a period of about one month without any appreciable loss of titre, and also when kept at 22° C. for short periods of 3 hours to 24 hours have been found also to maintain their potency.

A comparison was made of the relative stabilities of purified virus and unpurified virus in broth, 1 p.c. peptone water, and buffered phosphate solution, all at pH 7·6. Purified virus and stock filtrate were added respectively in a dilution of 1 : 100 to the three different media. Quanta of the different liquids were tested at intervals (*v.* Table IV). Purified virus suspended in broth was non-infective after 24 hours, and in peptone water after 5 days, while the same material suspended in buffered phosphate solution was found to be infective when tested on the 5th day. Unpurified virus remained infective in all three media for at least 5 days.

TABLE IV.—*Stability of Purified Virus Compared with that of Unpurified Virus in Broth, Peptone Water and* M/45 *Phosphate Buffer.*

Virus.	Time interval.	Medium.			Remarks.
		Broth.	*M*/45 phosphate buffer.	Peptone water.	
Purified virus	24 hours	00	++	+0	Temperature = 22° C.
	48 „	00	++	+0	Dilution of virus in different media = 1 : 100.
	5 days	00	++	00	pH of media = 7·6.
Unpurified virus (stock filtrate)	24 hours	++	++	++	++ = Both guinea-pigs inoculated showed lesions.
	48 „	++	++	++	+0 = One of two guinea-pigs inoculated showed lesions.
	5 days	++	++	++	00 = Neither of two guinea-pigs inoculated showed lesions.

Relative stabilities of purified virus in different broth media.—In the next series of experiments, purified virus was diluted 1 : 50 in different preparations of broth media from which quanta were subsequently removed at intervals, to be tested for infectivity. The results given in Table V show generally that the purified virus became non-infective more quickly at 22° C. in those broths in which the tissue proteins were partially digested than in those media in which no digestion of the tissue proteins had occurred, *e. g.* meat extract, or in broth in which complete digestion of the tissue proteins had taken place, viz. extra-digested broth which gave a negative reaction in the test for proteoses. This suggests that the substance in broth most harmful to the virus is some intermediate product of protein digestion. The ferment employed in the digestion process did not appear to affect the results. It is interesting to note that the purified virus survived as long in Martin's broth as in undigested meat extract.

The stabilizing influence of 25 mμ. *ultrafiltrate on the survival of purified virus in broth.*—Purified virus was diluted 1 : 50 in five different media, viz. (1) stock digest broth, (2) stock digest broth with equal parts of buffered phosphate solution, (3) stock digest broth with equal parts of the 25 mμ.

ultrafiltrate obtained from the primary washing of unpurified virus, (4) 25 mμ. ultrafiltrate, and (5) buffered phosphate solution. The different systems were kept at 22° C., and samples removed at intervals to be tested for infectivity. The results are given in Table VI. It was found that the virus suspended in broth and in broth with equal parts of buffered phosphate solution was non-infective after 20 hours, whereas virus suspended in buffered phosphate solution 25 mμ. ultrafiltrate and broth plus 25 mμ. ultrafiltrate remained infective for at least 5 days. This showed that the purified virus was protected against the harmful effect of the broth medium by the addition of the ultrafiltrate containing tissue proteins.

TABLE V.—*Relative Stabilities of Purified Virus in Different Broth Media.*

Medium.	Time intervals.			
	2 hours.	24 hours.	48 hours.	5 days.
Undigested meat extract (horse flesh).	++	++	++	00
(1) Digest broth [pepsin] (horse flesh)*.	++	++	00	00
(2) Digest broth [papain] (horse flesh)*.	++	++	+0	00
(3) Digest broth [trypsin] (horse flesh)*	++	++	00	00
Stock peptone broth	++	+0	00	00
Stock Lemco broth	++	++	+0	00
Stock digest broth (horse flesh)	++	00	00	00
Stock digest broth (ox heart)	++	00	00	00
Stock digest broth (rabbit flesh)	++	00	00	00
Martin's broth	++	++	++	—
Extra-digested broth (horse flesh)†	++	++	+0	00
M/45 buffered phosphate solution	++	++	++	++
Tap-water (London)	++	++	++	—

Temperature = 22° C.
Broths (digest) marked * were less completely digested than "stock" broth.
pH of all media = 7·6.
† Extra-digested broth gave a negative reaction in the proteose test.
Dilution of purified virus in different media = 1 : 50.

++ = Both of two guinea-pigs inoculated showed lesions.
+0 = One of two guinea-pigs inoculated showed lesions.
00 = Neither of two guinea-pigs inoculated showed lesions.
— = No test made.

TABLE VI.—*The Stabilizing Influence of 25 mμ. Ultrafiltrate on the Survival of Purified Virus in Broth.*

Medium.	Time intervals.			
	2 hours.	20 hours.	40 hours.	5 days.
Stock digest broth (horse flesh)	++	00	00	00
Stock digest broth (horse flesh) + *M*/45 phosphate buffer	++	00	00	00
Stock digest broth (horse flesh) + ultrafiltrate (25 mμ.)	++	++	++	++
Ultrafiltrate (25 mμ.)	++	++	++	++
M/45 phosphate buffer solution	++	++	++	++

Temperature 22° C.
Dilution of virus in different media = 1 : 50.
pH of media = 7·6.
++ = Both guinea-pigs inoculated showed lesions.

+0 = One of two guinea-pigs inoculated showed lesions.
00 = Neither of two guinea-pigs inoculated showed lesions.

Influence of pH on the stability of purified virus.—Experiments on the stability of purified preparations of foot-and-mouth disease virus at the critical pH values 3, 6 and 7·6 indicated that, as in the case of the unpurified virus (Pyl, 1934 ; Pyl and Klenk, 1933 ; Galloway and Elford, 1935), two zones of stability may be demonstrated, separated by a region in which the virus rapidly becomes inactivated. However, a sufficient number of experiments have not been made to give definite evidence as to whether or not any slight alteration in the relative stability is shown by the washed virus, but certainly there appears to be no pronounced displacement of the zones of stability.

PHOTODYNAMIC ACTION OF METHYLENE-BLUE ON PURIFIED VIRUS AND UNPURIFIED VIRUS.

It has been shown previously that the virus of foot-and-mouth disease is more resistant to inactivation by the photodynamic action of methylene blue than some other viruses.

Thus louping-ill virus, when in filtered or unfiltered suspensions, can be inactivated in five minutes with a 1 : 50,000 dilution of methylene-blue and employing as a source of light a 100 c.p. pointolite at a distance of 20 cm. (Perdrau and Todd, 1933). On the other hand, foot-and-mouth disease virus, as contained in stock membrane filtrates of broth suspensions of vesicle fluid, is not regularly inactivated even after one hour's irradiation with a 1 : 50,000 dilution of methylene-blue, and employing a 300 c.p. filament lamp at a distance of 13 cm. (Galloway, 1936).

In the present series of experiments the sensitiveness of purified virus in *M*/45 phosphate solution buffered at pH 7·6 to the photodynamic action of methylene-blue was studied and compared with that of unpurified virus, under the same conditions. A 300 c.p. filament lamp at a distance of 15 cm. was employed as a source of light. The final dilution of the dye was 1 : 50,000, and the virus (purified or unpurified) with the added dye was exposed in a layer 2 mm. thick in a small Petri dish. The virus was protected from the heat rays emitted by the lamp by the interposition of a glass vessel through which cold water ran continuously, a constant depth of 5 cm. of water being maintained throughout. The Petri dishes containing the virus-dye mixtures were placed over a cooling waterjacket, the upper surface of which was a polished plate of aluminium—a good conductor of heat. This is the method of irradiation elaborated by Perdrau and Todd since the publication of their first paper (*loc. cit.*).

Two experiments were made with purified virus preparations having a titre of between 1 : 10,000 and 1 : 100,000, and one with unpurified virus of equivalent titre. The results are given in Table VII (see p. 192). In one experiment purified virus was inactivated in 5 minutes, and in the other experiment in 10 minutes, while the unpurified virus was inactivated only after 45 minutes under the same conditions. Purified virus in the absence of methylene-blue was not rendered non-infective even after 45 minutes' irradiation, and another sample of purified virus with 1 : 50,000 methylene-blue was still infective added after remaining in the dark for the same period.

Relative protective action of 25 mμ. ultrafiltrate and serum : Experiment 1.—The conditions in this experiment were the same as described above. The standard time of irradiation was 15 minutes. Purified virus was diluted in seven different media, viz. (1) buffered phosphate solution, (2) 25 mμ. ultrafiltrate obtained from the primary washing of the virus, (3) 9 mμ. ultrafiltrate obtained by filtration of the 25 mμ. ultrafiltrate through a membrane A.P.D. 9 mμ. which holds back the serum proteins, (4) rabbit serum, (5) guinea-pig serum, (6) a filtrate of a suspension of rabbit brain, and (7) a filtrate of a suspension of guinea-pig brain. The results of the experiment are given in Table VIII, Experiment 1. It will be seen that the addition of 25 mμ. ultrafiltrate, serum (guinea-pig or rabbit), or filtrates of brain tissue (guinea-pig or rabbit), gave relative protection to the purified virus against the photodynamic action of methylene-blue. The 9 mμ. ultrafiltrate had no protective effect.

TABLE VIII.—*Photodynamic Action of Methylene-blue on Purified Virus ; Protective Action of 25 mμ. Ultrafiltrate and Serum.*

Experiment I. Liquid irradiated.	Test.	Remarks.
1·5 c.c. *M*/45 buffered phosphate solution + 0·5 c.c. virus	00	U.F. (A.P.D. 25 mμ.) = Ultrafiltrate employing membrane of average pore diameter 25 mμ. allowing passage of serum proteins.
1·5 c.c. U.F. (A.P.D. 25 mμ.) + 0·5 c.c. virus	++	U.F. (A.P.D. 9 mμ.) = ultrafiltrate employing membrane of average pore diameter 9 mμ. holding back serum proteins.
1·5 c.c. U.F. (A.P.D. 9 mμ.) + 0·5 c.c. virus	00	Concentration of methylene-blue = 1 : 50,000.
1.5 c.c. rabbit serum + 0·5 c.c. virus	++	Time of irradiation = 15 mins.
1·5 c.c. guinea-pig serum + 0·5 c.c. virus	++	++ = Both guinea-pigs inoculated showed lesions.
1·5 c.c. filtrate of rabbit brain + 0·5 c.c. virus	+0	+0 = One of two guinea-pigs inoculated showed lesions.
1·5 c.c. filtrate of guinea-pig brain + 0·5 c.c. virus	+0	00 = Neither of two guinea-pigs inoculated showed lesions.
Experiment II.		
Virus in *M*/45 phosphate irradiated through guinea-pig serum	00	
Virus in *M*/45 phosphate irradiated through U.F. (A.P.D. 25mμ.)	00	
Virus in *M*/45 phosphate irradiated through *M*/45 phosphate	00	

Experiment 2.—In order to determine whether this "protective" action of 25 mμ. ultrafiltrate or serum might simply be due to the absorption of a portion of the active light, purified virus suspended in phosphate solution buffered *M*/45 at pH 7·6 and with 1 : 50,000 methylene-blue added was exposed to light which first passed through a layer of serum or 25 mμ. ultrafiltrate. The mixtures of virus with methylene-blue were placed in dark cells made by painting the outside of the Petri dishes with black lacquer and covering this again with black paper (Fig. 2). Above these cells were placed other small dishes containing a 2-mm. layer of serum, 25 mμ. ultrafiltrate or buffered phosphate solution. The only light reaching the mixtures was therefore that which passed through the upper layers of liquid. The results of the experiment are given in Table VIII, Experiment 2. The purified virus in the presence of methylene-blue was inactivated in 15 minutes even when the light passed

through a layer of serum, 25 mμ. ultrafiltrate or buffered phosphate solution. This demonstrated quite definitely that the protective action of serum, or ultrafiltrate containing serum proteins, is not due to specific absorption of light by these substances, but is only manifest when the serum proteins are actually mixed with the purified virus subjected to irradiation in the presence of methylene-blue. One experiment was made with unpurified virus under the same conditions as described above, the infective filtrate being irradiated through a layer of buffered phosphate solution 2 mm. thick. The unpurified virus was still infective at the end of the irradiation time of 15 minutes.

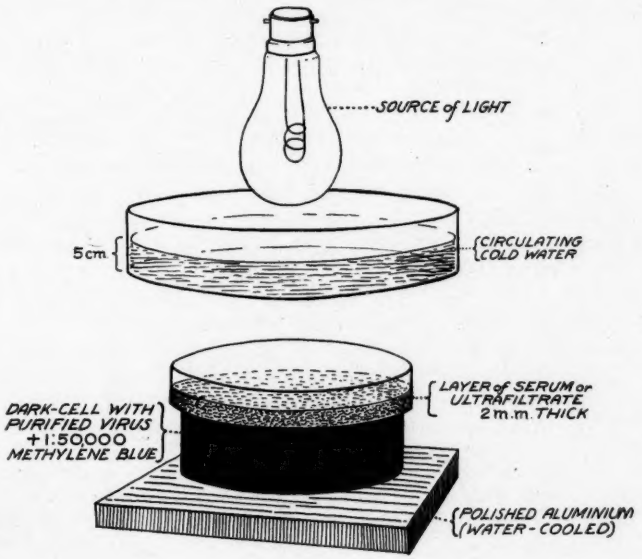
SOURCE of LIGHT
5cm.
CIRCULATING COLD WATER
LAYER of SERUM or ULTRAFILTRATE 2 m.m. THICK
DARK-CELL WITH PURIFIED VIRUS +1:50000 METHYLENE BLUE
POLISHED ALUMINIUM (WATER-COOLED)

FIG. 2.

Experiment 3.—A series of mixtures of purified virus and different concentrations varying from 75 p.c. to 10 p.c. of guinea-pig serum or 25 mμ. ultrafiltrate were made. The concentration of virus was made the same in each case by addition of the requisite amount of $M/45$ buffered phosphate solution. Sufficient methylene-blue solution was added to make the final concentration of the dye 1 : 50,000. Then each mixture was irradiated under the same conditions for 15 minutes. The results are given in Table IX. Under the conditions of the experiment, the addition of 10 p.c. guinea-pig serum to the purified virus was sufficient to protect it against the photodynamic action of methylene-blue, while the addition of 50 p.c. ultrafiltrate was necessary to obtain the same result.

As already pointed out, the sulphosalicylic acid test for protein gives a slightly positive reaction with ultrafiltrate diluted 1 : 100. A series of dilutions of guinea-pig serum were made, and the sulphosalicylic acid test applied to

each dilution. A slightly positive reaction was given with a 1 : 10,000 dilution of serum, but not with a 1 : 100,000. The filtrates of brain tissue employed in Experiment 1 (*v. supra*) gave a positive reaction in a dilution of 1 : 100. That a lesser amount of guinea-pig serum was necessary in the above experiment to protect the purified virus can therefore be explained by the greater concentration of serum proteins.

TABLE IX.—*Photodynamic Action of Methylene-blue on Purified Virus; Protective Effects of 25 mμ. Ultrafiltrate and Serum.*

Protective agent.	Concentration. 75 p.c.	50 p.c.	25 p.c.	10 p.c.	Control: no protective agent.
Guinea-pig serum	++	++	++	++	00
Ultrafiltrate (A.P.D. 25 mμ.)	++	++	00	00	00

Remarks.

Time of irradiation = 15 mins.
++ = Virus present. Both guinea-pigs inoculated showed lesions.
00 = No virus present. Neither of two guinea-pigs inoculated showed lesions.
Concentration of methylene-blue = 1 : 50,000.

Similar experiments were made with guinea-pig serum and ultrafiltrate heated to 60° C. for 30 minutes, and again relative protection of purified virus against inactivation by the photodynamic action of methylene-blue was observed. The results point to the necessity of examining purified preparations of virus in estimating the real degree of sensitiveness of any given virus to the photodynamic action of methylene-blue. In the case of foot-and-mouth disease virus at least it has been shown that the apparent resistance can be explained by the protective action of the serum proteins present in the infective vesicle lymph.

EFFECT OF ALCOHOL ON PURIFIED VIRUS COMPARED WITH ITS EFFECT ON UNPURIFIED VIRUS, *B. COLI*, *STAPHYLOCOCCUS*, AND THE MICRO-ORGANISM OF BOVINE PLEURO-PNEUMONIA.

The resistance of the virus of foot-and-mouth disease to different concentrations of alcohol has been studied at ordinary laboratory temperatures by several workers, notably by Abe (1925), Bedson and Maitland (1925), and Olitsky and Boëz (1927). The two former groups of investigators concluded that the virus in unfiltered diluted vesicle lymph is much more resistant to 60 p.c. alcohol than are micro-organisms such as *Staphylococcus*. In one experiment Bedson found that the virus resisted the action of 60 p.c. alcohol for 18 hours at room temperature. His results were, however, irregular, and in other tests the virus was inactivated in very much shorter periods. *Staphylococcus* was inactivated in 1 minute under the same conditions. Olitsky and Boëz, on the other hand, who examined not only unfiltered virus suspensions, but also Berkefeld filtrates, concluded that "the viewpoint that the virus of foot-and-mouth disease by itself is more resistant to alcohol than bacteria is incorrect". They found that the virus in unfiltered diluted lymph resisted 60 p.c. alcohol for a period of about 24 hours at ordinary room temperature.

Virus in Berkefeld filtrates, however, was inactivated in a few minutes under the same conditions, *i. e.* according to their results, in about the same time as *Staphylococcus*.

In the experiments just described 90 p.c. alcohol was added to the unfiltered virus suspensions or filtrates until the required concentration of alcohol, viz. 60 p.c., was reached. During the addition of 90 p.c. alcohol to diluted unfiltered or filtered virulent vesicle lymph, precipitation occurs almost immediately, and this may or may not be partially reversible according to whether unfiltered or filtered virus is employed. In testing for infectivity the irreversible precipitate (washed or unwashed), and the supernatant fluid or the precipitate resuspended in alcohol was inoculated intradermally into metatarsal pads of guinea-pigs. Such experimental methods would be likely to lead to irregular results, and moreover to no true estimate of the actual resistance of the virus to alcohol.

Experimental methods employed by the present writers.—66·6 p.c. and 33·3 p.c. concentrations of alcohol in buffered phosphate solution at pH 7·6 were prepared. To 9 c.c. amounts of each of these concentrations of alcohol, 1 c.c. of purified virus or unpurified virus of equal potency was added, so that the final concentrations of alcohol were 60 p.c. and 30 p.c. No precipitate could be detected in any of the mixtures prepared by this technique. Experiments were made at three different temperatures, 22° C., 0° C. and −10° C. In testing the infectivity of the different fluids, 0·5 c.c. was removed from each and diluted in 4·5 c.c. of buffered phosphate solution, so that the concentration of alcohol was reduced to 6 p.c. or 3 p.c. before inoculation. Each of two guinea-pigs was inoculated intradermally into the pads, and at the same time with 2·5 c.c. of the fluid intraperitoneally.

In the tests with *B. coli* and *Staphylococcus* similar methods were employed. Standard suspensions of these micro-organisms were made in broth such that viable bacteria could still be demonstrated by subculture into broth in dilutions of about 10^{-7} to 10^{-8}. The " titre " of these bacterial suspensions was thus greater than those of unpurified or purified virus suspensions, viz. 10^{-5}. The conditions of the comparative tests might be considered, therefore, to favour the bacteria rather than the virus. On the other hand, the cultural test for the viability of bacteria is probably more sensitive than the animal test for virus, since we have no knowledge of the number of virus particles constituting the minimum infective dose. In testing for the viability of the bacteria subjected to the action of alcohol, 0·5 c.c. amounts of the mixtures were subcultured at intervals into about 25 c.c. of broth. This procedure reduced the concentration of the alcohol in the subculture tubes to less than 6 p.c. From the results of preliminary tests this reduction of the concentration of alcohol was found to be a very necessary precaution, since in control tests, if 0·5 c.c. quanta of 60 p.c. alcohol were inoculated into only about 5 c.c. of broth, the resultant concentration of the alcohol, viz. 6 p.c., was found to be sufficient to inhibit the growth of *Staphylococcus*. In actual tests growth in subculture tubes was sometimes obtained when the test samples were inoculated into 25 c.c. of broth, and no growth occurred when only 5 c.c. amounts of broth were employed.

Dr. F. F. Tang very kindly made the tests on the micro-organism of bovine

pleuro-pneumonia. Two different cultures of the organism were employed, one a recently isolated or "young" strain, from a case of the disease in Shanghai, known as "Abattoir I", and the other an "old" laboratory strain, "Lister". The differences observed between the cultural characteristics of "young" and "old" strains have been referred to in previous papers (Tang, Wei, McWhirter and Edgar, 1935; Tang and Edgar, 1936).

The method of making the tests with the pleuro-pneumonia organism was similar to that employed for *B. coli* and *Staphylococcus*. The precaution of reducing the concentration of the alcohol in the subculture tubes to less than 6 or 3 p.c. was found to be even more necessary than with the bacteria tested, since alcohol, especially in a concentration of 6 p.c., has a definite inhibitory action on the growth of the organism. The pleuro-pneumonia organism grows only in broth to which about 10 p.c. of serum has been added, so that when 1 c.c. of a serum broth culture was added to 9 c.c. of 66·6 or 33·3 p.c. alcohol, a definite precipitate was formed. This was resuspended before the samples were removed for making the subculture tests.

The results of the experiments are given in Table X.

Two different experiments with each concentration of alcohol were made in the cases of purified virus, unpurified virus, *B. coli* and *Staphylococci*. Both purified and unpurified virus behaved very similarly as regards sensitiveness to the action of 60 p.c. alcohol at 22° C.—both survived for about 5 to 15 minutes, but not 30 minutes. In preliminary tests similar results were

TABLE X.—*Comparison of the Effect of Alcohol on Purified Virus and Micro-organism of Bovine Pleuro-pneumonia.*

	Percentage of alcohol.	Experiment No.	22° C. 1 min.	2 min.	3 min.	4 min.	5 min.	10 min.	15 min.	30 min.	1 hour
Purified virus	60	1	—	—	—	—	++	—	00	00	00
		2	—	—	—	—	++	+0	+0	00	00
	30	1	—	—	—	—	++	++	++	++	++
		2	—	—	—	—	++	++	++	++	++
Unpurified virus	60	1	—	—	—	—	++	—	00	00	00
		2	—	—	—	—	++	++	00	00	00
	30	1	—	—	—	—	++	++	++	++	++
		2	—	—	—	—	++	++	++	++	++
B. coli	60	1	+	—	—	—	0	0	0	0	0
		2	0	0	0	0	0	—	—	—	—
	30	1	—	—	—	—	+	+	+	+	+
		2	—	—	—	—	+	+	+	+	+
Staphylococcus	60	1	0	—	—	—	0	0	0	0	0
		2	0	0	0	0	0	0	0	0	0
	30	1	—	—	—	—	+	+	+	+	+
		2	—	—	—	—	+	+	+	+	+
Bovine pleuro-pneumonia (B.P.P.)	60	"Lister"	0	—	—	—	0	0	0	0	0
		"Abt. I"	0	—	—	—	0	0	0	0	0
	30	"Lister"	+	—	—	—	0	0	0	0	0
		"Abt. I."	+	—	—	—	+	0	0	0	0

— = No test made.
++ = Both guinea-pigs of two inoculated showed lesions.
+0 = One guinea-pig of two inoculated showed lesions.
00 = Neither of two guinea-pigs inoculated showed lesions.

obtained with diluted and well-centrifuged but unfiltered fresh, virulent vesicle lymph. The results are not shown in the table.

Unpurified virus was not inactivated even after 18 hours by 30 p.c. alcohol at 22° C., whereas purified virus survived 2 but not 3 hours. At 0° C. purified virus remained infective in 60 p.c. and 30 p.c. alcohol for 18 hours, but not 4 days. Unpurified virus remained infective for at least 7 days in both concentrations of alcohol under the same conditions. At — 10° C. the purified virus remained viable for 4 days but not 7 days in both 60 p.c. and 30 p.c. alcohol, and the unpurified virus for at least 7 days in both concentrations.

B. coli and *Staphylococcus* were more sensitive to the action of 60 p.c. alcohol at 22° C. than even the purified virus. The difference in sensitiveness between the purified virus and the bacteria to this concentration of alcohol was not so noticeable at low temperatures. There was very little difference in the susceptibility of purified virus, *B. coli* and *Staphylococcus* to the lethal action of 30 p.c. alcohol.

The micro-organism of pleuro-pneumonia, especially taking into account the fact that it was suspended in a serum broth medium, was more sensitive to the lethal action of 60 p.c. alcohol than *B. coli* or *Staphylococcus*. The results at 22° C. showed that the pleuro-pneumonia organism was also more sensitive than the latter bacteria to the lethal action of 30 p.c. alcohol, the "Lister" or "old" strain being slightly more sensitive than the "Abattoir" or "young" strain.

Purified Virus, Unpurified Virus, B. coli, Staphylococcus *and the Pleuro-pneumonia.*

						0° C.					—10° C.				
C.		1 hour.	2 hours.	3 hours.	6 hours.	18 hours.	18 hours.	36 hours.	4 days.	7 days.	18 hours.	36 hours.	4 days.	7 days.	
In.	30 min.														
	00	00	00	00	00	00	++	—	00	00	++	—	+0	00	
	00	00	00	00	00	00									
+	++	++	++	00	00	00	++	—	00	00	++	—	++	00	
+	++	++	+0	00	00	00									
	00	00	00	00	00	00	++	—	++	++	++	—	++	++	
	00	00	00	00	00	00									
+	++	++	++	++	++	++	++	—	++	++	++	—	++	++	
-	++	++	++	++	++	++									
	0	0	0	0	0	0	+	0	—	—	+	0	—	—	
	—	—	—	—	—	—									
	+	+	0	0	0	0	+	+	—	—	+	+	—	—	
	+	+	0	0	0	0									
	0	0	0	0	0	0	0	0	—	—	+	0	—	—	
	0	0	0	0	0	0									
	+	+	0	0	0	0	+	+	—	—	+	+	—	—	
	+	+	0	0	0	0									
	0	0	0	0	0	0	0	—	—	—	0	—	—	—	
	0	0	0	0	0	0	0				0				
	0	0	0	0	0	0	+	—	—	—	+	—	—	—	
	0	0	0	0	0	0	+				+				

+ = Growth of micro-organism.
0 = No growth of micro-organism.
"Lister" is an "old" culture of B.P.P.
Abt. is a "young" culture of B.P.P.

The results of these experiments show generally that unpurified foot-and-mouth disease virus is more resistant to the action of alcohol than the purified virus from which the serum proteins have been removed. The degree of resistance of the virus is much less than is generally supposed, and can be explained in part at least by the protective action given by the serum proteins with which it is associated in unpurified preparations. The virus, even in purified preparations, appears to be a little more resistant to the lethal action of 60 p.c., especially at 22° C., than bacteria such as *B. coli*, *Staphylococcus* and the micro-organism of bovine pleuro-pneumonia.

FILTERABILITY OF PURIFIED VIRUS COMPARED WITH THAT OF UNPURIFIED VIRUS.

The virus of foot-and-mouth disease in an unpurified state, when suspended in a phosphate medium buffered at 7·6, filters less easily than when it is suspended in digest broth (Galloway and Elford, 1931). Thus the virus when suspended in a broth will pass through all gradocol membranes down to the filtration end-point A.P.D. 25 mμ., but when suspended in a buffered phosphate medium is retained by all membranes below about A.P.D. 60 mμ. The results of filtration tests made with purified virus suspended in buffered phosphate solution showed that it behaved in the same way as unpurified virus, and had no tendency to filter more easily. It was not possible to make comparative experiments with purified virus suspended in broth, since, as explained above, it is not stable in this medium.

RESISTANCE OF PURIFIED VIRUS TO DRYING.

A 1 c.c. amount of purified virus was placed in a vaccine phial and dried *in vacuo*. The first part of the drying process was effected over H_2SO_4, and then more complete drying carried out over P_2O_5. The dried, purified virus was kept for 8 days *in vacuo*, and proved still to be infective at the end of this period.

SUMMARY.

It has been found possible to make preparations of purified foot-and-mouth disease virus in buffered phosphate solution at pH 7·6 by repeated washing on collodion membranes of suitable porosity. These preparations gave a negative reaction in the test for proteins (sulphosalicylic acid test), and had a potency equivalent to the stock filtrates.

The stability of purified virus in buffered phosphate solution over a period of about 3 days at 22° C., or one month at 0° C. compared very favourably with that of unpurified virus. After much longer periods the loss in titre of the purified virus was somewhat greater than that of unpurified virus kept under the same conditions. Slight differences were observed in the stabilities of the various preparations of purified virus, doubtless attributable to varying degrees of " purity " below the limiting concentration of protein detectable by the sulphosalicylic acid test.

The purified virus differed from unpurified virus in (1) its relative instability in a digest broth medium, (2) its greater sensitiveness to the photodynamic action of methylene-blue, and (3) its greater susceptibility to the lethal action of certain concentrations of alcohol.

The filtration behaviour of the purified virus suspended in a buffered phosphate medium at pH 7·6 was in every way similar to that of unpurified virus under the same conditions.

Tests on the survival of purified foot-and-mouth disease virus at the critical pH values 3, 6 and 7·6 showed that, as in the case of the unpurified virus, two zones of relative stability may be demonstrated, separated by a region in which the virus rapidly becomes inactivated. The number of experiments made was not sufficient to decide definitely whether or not any slight alteration in relative stability is shown by the washed virus, but certainly there appeared to be no pronounced displacement of the pH zones of stability.

Purified virus dried *in vacuo* remained infective for at least a week at − 4° C. to 0° C.

REFERENCES.

ABE, T.—(1925) *Z. InfektKr. Haust.*, **28**, 111.

BARNARD, J. E., AND ELFORD, W. J.—(1931) *Proc. Roy. Soc.*, B, **109**, 360.

BEDSON, S. P.—(1932) *Brit. J. exp. Path.*, **13**, 65.

Idem AND MAITLAND, H. B.—(1925) *J. comp. Path.*, **38**, 238.

CRAIGIE, J.—(1932) *Brit. J. exp. Path.*, **13**, 259.

DUBOS, R., AND BAUER, J. H.—(1935) *J. exp. Med.*, **62**, 271.

ELFORD, W. J., AND ANDREWES, C. H.—(1932) *Brit. J. exp. Path.*, **13**, 446.—(1935) *Ibid.*, **16**, 61.

GALLOWAY, I. A.—(1936) In press. *5th Progress Report of the Foot-and-Mouth Disease Research Committee*, H.M. Stationery Office, London.

Idem AND ELFORD, W. J.—(1931) *Brit. J. exp. Path.*, **12**, 407.—(1935) *Ibid.*, **16**, 588.

JANSSEN, L. W.—(1936) "*Over Mond-en Klauwzeervirus*," Staatsveeartsenijkundig Onderzoekingsinstituut, Rotterdam. 'S-Gravenhage-Algemeene Landsdrukkerij.

OLITSKY, P. K., AND BOËZ, L.—(1927) *J. exp. Med.*, **45**, 815.

PARKES, R. F., AND RIVERS, T. M.—(1935) *Ibid.*, **62**, 65.

PERDRAU, J. R., AND TODD, C.—(1933) *Proc. Roy. Soc.*, B, **112**, 288.

PYL, G.—(1934) *Hoppe-Seyler's Z.*, **226**, 18.

Idem AND KLENK, L.—(1933) *Zbl. Bakt.*, Abt. I, Orig. **128**, 161.

SCHLESINGER, M.—(1933) *Biochem. Z.*, **264**, 6.

TANG, F. F., WEI, H., MCWHIRTER, D. L., AND EDGAR, J.—(1935) *J. Bact.*, **40**, 391.

TANG, F. F., AND EDGAR, J.—(1936) *Ibid.*, **42**, 45.

THE PRODUCTION OF ANTISERA WITH SUSPENSIONS OF POTATO VIRUS " X " INACTIVATED BY NITROUS ACID.

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VARIOUS workers have shown that antibodies reacting specifically with sap from plants infected with certain viruses can be produced in rabbits. There is a large amount of evidence indicating that the specific antigens are the viruses (or parts of them), but this cannot be taken as proved until the viruses have been obtained free from other materials. The serological reactions of the virus suspensions appear to be independent of the species of the host-plant, and are not affected by the removal of the greater part of the plant proteins. With potato virus " X " Spooner and Bawden (1935) showed that filters which remove the virus also remove the antigen. Bawden (1935) found that a close quantitative relationship exists between the antigen content of suspensions of the S strain of virus " X ", as measured by their optimal flocculation points, and the virus content as measured by their capacity to produce local lesions. It was also found that the antigen and virus behave similarly with certain precipitants, and that inactivation of the virus by heat, ageing *in vitro* and alcohol is accompanied by a corresponding loss of reactions with anti-viral serum. Bawden and Pirie (1936) further showed that the loss of infectivity following on pH changes and exposure to certain proteolytic enzymes is also accompanied by loss of power of flocculation with anti-viral serum. On the other hand, Bawden (1935) showed that suspensions of virus " X " treated with formaldehyde lose their infectivity but still flocculate unchanged with antiserum, and Bawden and Pirie (1936) showed that suspensions of virus "X" treated with nitrous acid behave similarly.

We have now found that suspensions of virus " X " inactivated by nitrous acid not only retain their power of reacting with anti-viral serum, but they are still antigenic, and when injected intravenously into rabbits produce antisera indistinguishable from those produced by injecting highly infective

suspensions. We are unaware of any previous use of nitrous acid as an inactivating agent, and do not know if it has a similar action on any pathogens other than potato virus "X". Lewis (1934), however, has shown that the antigenic behaviour of casein is unaffected by treatment with nitrous acid.

Materials and methods.—The purified suspensions used were pale brown and water clear, and were prepared in the manner described by Bawden and Pirie (1936). The suspensions were adjusted to pH 4 by the addition of $N/10$ HCl and then divided into equal parts, one of which was kept as the control virus suspension. The other was cooled to 0° C., and to it was added enough of a strong solution of $NaNO_2$, previously adjusted to pH 4 with acetic acid, to give a final concentration of 1·5 p.c. $NaNO_2$ in the nitrite-treated suspensions. After half an hour at 0° C. both suspensions were thoroughly dialysed, and then made up to the same volume by the addition of distilled water.

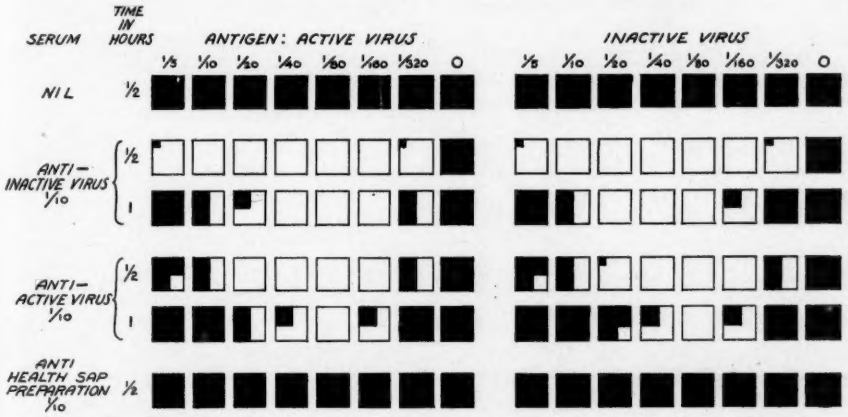

FIG. 1.—Complement-fixation by active virus suspension and virus suspension inactivated by nitrous acid with antisera against both and against healthy tobacco sap preparation. Hæmolytic amboceptor at three times its minimal hæmolytic strength with the dose of complement used. Fixation allowed to proceed for 1 hour.

Before injection into rabbits the suspensions were tested both for their reactions with antisera and for infectivity. The technique used in these tests was similar to that described in previous papers. The control and nitrite-treated suspensions both flocculated and fixed complement with antiviral sera, but not with the sera of rabbits injected with sap from healthy tobacco plants, but whereas 45 tobacco plants inoculated with control virus suspensions all showed numerous local lesions and later developed systemic symptoms, 64 plants inoculated with nitrite-treated suspensions all remained healthy.

It was found that a single intravenous injection of 2 c.c. of these purified virus suspensions yielded potent sera which were almost as good as those previously obtained after a long series of intraperitoneal injections of virus-containing sap. This intravenous method of immunization was therefore used, and the rabbits were bled from the ear ten days after one injection. Two rabbits received nitrite-treated virus suspensions, and two control virus

suspensions. Two more were treated in the same way with the product obtained by subjecting the sap of healthy tobacco plants to the purification process. This healthy plant preparation did not flocculate with anti-viral serum, and 30 tobacco plants inoculated with it remained healthy.

Results.—A large number of experiments has failed to show any significant difference between the sera of rabbits injected with the nitrite-treated virus suspensions and of those injected with the control suspensions of active virus. Both flocculated equally with either active virus suspensions or suspensions inactivated by nitrous acid, but not with the preparations from healthy plants. Both types of antiserum also fixed complement with both types of virus suspension. The sera from rabbits injected with the material from healthy plants gave no reaction with either active virus suspensions, suspensions inactivated by nitrous acid or with material from healthy plants. The results from one such experiment are shown in Table I and Fig. 1.

TABLE I.—*Flocculation Experiment with Virus and Healthy Plant Antigens and their Respective Antisera.*

Serum prepared against—	Dilution.	Antigens. Active virus suspension 1/20. (1-hour reading.)	Nitrite-treated inactive virus suspension 1/20. (1-hour reading.)	Healthy sap preparation 1/20. (24-hour reading.)
Active virus suspension	1/4	++++	++++	—
	1/8	++++	++++	—
	1/16	+++	+++	—
	1/32	++	++	—
	1/64	+	+	—
	1/128	—	—	—
	0	—	—	—
Nitrite-treated inactive virus suspension	1/4	++++	++++	—
	1/8	++++	++++	—
	1/16	++++	++++	—
	1/32	+++	+++	—
	1/64	++	++	—
	1/128	+	+	—
	0	—	—	—
Healthy sap preparation (all 24-hour readings).	1/4	—	—	—
	1/8	—	—	—
	1/16	—	—	—
	1/32	—	—	—
	1/64	—	—	—
	1/128	—	—	—
	0	—	—	—

+ signs indicate the degree of flocculation.

The sera obtained by injecting inactive, nitrite-treated virus suspensions were found to be as effective in neutralizing the virus *in vitro* as were the sera produced by injecting active virus suspensions. The tests were made by

mixing 1 c.c. of purified virus suspension with 1 c.c. of diluted antiserum. The mixtures were left at room temperature for two hours and then shaken thoroughly before inoculation to tobacco plants. The inoculations were made with ground-glass spatulæ, and the mixtures were rubbed as evenly as possible over the whole leaf surface. The results from several such experiments are summarized in Table II.

TABLE II.—*The Inactivation of Potato Virus "X" in vitro by Sera.*

Serum.	Dilution.	Number of plants inoculated.	Number of plants infected.	Percentage infected.
Normal rabbit	1/5	25	25	100
" "	1/10	10	10	100
Anti-healthy tobacco	1/5	12	12	100
" "	1/10	12	12	100
Anti-active virus	1/5	40	2	5
" "	1/10	25	5	20
Anti-nitrite treated virus	1/5	40	1	2
" " "	1/10	25	2	8

SUMMARY.

Antisera indistinguishable from those prepared by injecting suspensions of active virus can be prepared in rabbits by the intravenous injection of suspensions of virus "X" inactivated by nitrous acid. Both fix complement and flocculate with virus suspensions, but not with the sap of healthy tobacco plants, and both are equally effective in neutralizing the virus *in vitro*.

REFERENCES.

BAWDEN, F. C.—(1935) *Brit. J. exp. Path.*, **16**, 435.
Idem AND PIRIE, N. W.—(1936) *Ibid.*, **17**, 64.
LEWIS, J. H.—(1934) *J. Infect. Dis.*, **55**, 203.
SPOONER, E. T. C., AND BAWDEN, F. C.—(1935) *Brit. J. exp. Path.*, **16**, 218.

THE TYPHUS GROUP OF DISEASES IN MALAYA. PART IV: THE ISOLATION OF TWO STRAINS OF TROPICAL TYPHUS FROM WILD RATS.

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THE cumulative evidence from the investigations of Fletcher and his co-workers, of Anigstein and of Gater, suggests that the viruses of rural typhus and the tsutsugamushi disease share the only reservoir as yet known—the rat. Thus Fletcher, Lesslar and Lewthwaite (1928), in an examination of 130 rats trapped in endemic and non-endemic foci, found the larval mite, *Trombicula deliensis*, only in rats from endemic areas (with one exception); and, within such an endemic area, more frequently in a particular area in which most of the cases occurred.

Gater (1930) examined 743 wild rats, approximately one-half from the same endemic area noted above, and one-half from the town of Kuala Lumpur, month by month for one year. He reports: "Only 3 trombidiid mites known to attack man were found on the town rats, one specimen of each of three species being obtained. On the rural rats they were much more numerous: *T. deliensis* was found on 21·3 p.c.; *T. pseudoakumushi* on 7 p.c.; *T. akamushi* on 1·8 p.c.; and *T. acuscutellaris* Walch on 2 p.c." Further, he suggests that *T. akamushi* and *T. deliensis* are merely forms of the same species which may prove to be the carrier of both diseases. These two larval mites are respectively the carriers of the tsutsugamushi disease of Japan and the closely allied (if not identical) mite-fever of Sumatra. The *T. akamushi* was by far the commonest ectoparasite found by Gater on labourers in the endemic area.

This concentration of the probable vector on the rat population of an endemic area suggests the rat as a likely reservoir of the viruses.

Anigstein (1933) carried out Weil-Felix reactions on the sera of 130 wild rats caught in the endemic area noted above. In 1 the OX19 type of *Proteus* was agglutinated to a titre of 1/500, in 3 to one of 1/250; in 2 others the OXK type was agglutinated to a titre of 1/250. The significance of these agglutination reactions seems, however, to have been lessened through an observation recorded by Felix (1933), who states that "O agglutinins of relatively high titres for one or the other *Proteus* X type were often found in the serum of normal tame rats". According to a personal communication from Dr. Felix agglutination titres of 1 in 500 for the strains OX19 and OXK have been observed in normal tame rats of London stock. Anigstein (1933) also reported that in 200 rats from the same area 9 gave macroscopic evidence of a scrotal

reaction, and microscopic evidence of the presence of *Rickettsia*-like organisms in material from the tunica vaginalis.

In the case of urban typhus the authors have recently shown, in collaboration with Mr. E. P. Hodgkin, Entomologist to this Institute, and will report in a later paper, that the rat flea (*Xenopsylla cheopis*) can acquire the virus by feeding on an infected white rat; and that on transference to another white rat it can transmit the infection to this rat. Again, a considerable number of cases of urban typhus come from food and grain shops, in which rats abound; in other cases a history of house or office being infected with rats is almost invariably given.

The present authors have sought additional evidence on the problem of the reservoir of the virus. Twenty-three attempts were made to isolate, in the guinea-pig, strains of tropical typhus from wild rats trapped either in the particular houses or circumscribed rural areas in which cases of the infection had recently occurred. Three attempts succeeded. Maintenance of one strain was abandoned after the seventh generation, because of the heavy drain entailed of the supply of laboratory animals; two other strains, called the "Rat 4" strain and the "Rat 5" strain respectively, were studied more thoroughly.

THE "RAT 4" STRAIN.

This strain was initiated by the inoculation into a wild (brown) rat, caught at random in the town of Kuala Lumpur, of a suspension of brain, spleen and testis of a wild (brown) rat that had been trapped in a house (on the outskirts of the town) in which a case of urban typhus had recently occurred. The rat remained afebrile; on the 19th day after inoculation it was killed, a similar suspension made, and inoculated by the intra-peritoneal route into 2 guinea-pigs that had been fed on a diet deficient in vitamin A. A febrile and scrotal reaction resulted. By similar inoculations the strain was maintained in guinea-pigs for two years, with gradual enhancement of virulence. Whether the strain originated in the first or second of these two wild rats cannot be known with certainty. The authors have previously shown (Parts I and III of this series) that passage of the viruses of rural and urban typhus through the white rat enhances their virulence, although the infections are "inapparente"; and such an enhancement may well have occurred in this instance.

The Infection in the Guinea-pig.

After an incubation period of 5–12 days a marked febrile reaction of 8–11 days' duration occurred. The temperature rose during 2 or 3 days to a range of 104°–105° F., and defervescence occupied a like period. A large proportion of guinea-pigs died towards the end of the febrile reaction. In 70 p.c. of males inoculated by the intra-peritoneal route a swelling, redness and tenderness of the scrotum occurred, early signs of this reaction appearing occasionally on the day prior to onset of fever, usually coincidentally with that event, and, rarely, on the second or third day of fever. These two signs resulted whether the passage material were brain, spleen, blood or exudate from the tunica vaginalis. Where tunica exudate was used, the incubation period was shorter; the extreme virulence of this inoculum was shown by the fact that its injection

in very high dilutions still evoked febrile and scrotal reactions. Subcutaneous inoculation lengthened the incubation period and diminished the severity of the infection. In a minority of guinea-pigs inoculated by the subcutaneous route a mild scrotal reaction also occurred; redness and tenderness were rarely seen, and adhesions of the testis always resulted. Such a reaction following inoculation by this route has not been seen in experimental infections with strains of human origin (*vide* Part III), and was probably due to the presence of the *Spirillum morsus muris* as a secondary invader (*vide infra*).

Post-mortem findings have been constant, and characteristic of infections with a typhus virus, viz. injection of the vessels of the visceral and parietal layers of the tunica vaginalis, and hæmorrhages on their surface and into the polar fat; increase of fluid in the tunica sac, enlargement of the lymphatic glands, frequently with hæmorrhage into their substance; a deposit of fibrin on the surface of an enlarged spleen; and, occasionally, subpleural hæmorrhages and hyperæmia of the suprarenal glands. Examination of Giemsa-stained smears of the tunica exudate and of the fibrin from the surface of the spleen revealed within endothelial cells large numbers of *Rickettsia*, short, rod-shaped, bipolar-staining organisms. In morphology, staining characteristics, distribution and numbers they did not differ from those described in detail by the authors (1936, Parts I and II) in the experimental infections of guinea-pigs and rabbits with the virus of strains of rural typhus and the tsutsugamushi disease derived from human sources. They thus resemble *R. orientalis* rather than *R. prowazeki*.

As noted above, the guinea-pigs of the early generations were fed on a vitamin-deficient diet; and comparison with a collateral series of inoculated guinea-pigs fed on an ordinary diet showed the febrile reaction to be more regular and the scrotal reaction to be of much more frequent occurrence in the former series. Later, as the strain became more firmly established, it was found that the vitamin-deficient diet could be discontinued without risk of losing the strain.

The Infection in the Rabbit.

Further evidence of the typhus-like nature of the infection with the "Rat 4" strain was afforded by the Weil-Felix reaction in rabbits inoculated with the virus by both the intra-peritoneal and the intra-ocular routes. Sera were tested before inoculation, and repeatedly throughout convalescence for 60 days. From 23 rabbits thus inoculated, 17 sera were positive, titres exceeding 1/400 in 5, and in 2 being as high as 1/770 and 1/1700. Agglutination was invariably of the OXK type of *Proteus* only (for a detailed analysis of the serological reactions see p. 218).

In 13 instances attempts were made to isolate strains in the rabbit by the inoculation of virus by the intra-ocular route—a method of inoculation successful in the case of the viruses of rural typhus and the tsutsugamushi disease (see Parts I and II), and also, as reported below, in the case of the other of these two rat strains. Heart-blood or tunica exudate, taken from a passage guinea-pig at the height of reaction, was inoculated into one or both eyes; in 2 rabbits only did any trace of an ocular reaction result; and it could not

be reproduced in a second generation of rabbits following intra-ocular injection of aqueous humour. Yet from 8 rabbits thus inoculated the sera gave a positive Weil-Felix reaction of the OXK type, from 2 to very high titres.

Thus the virus of a strain isolated from one of two rats caught in an urban area proved to be XK in type, not X19 as was to have been expected. But in Kuala Lumpur, as in most Malayan towns, countryside encroaches extensively on town, making considerable migration of rats probable in the outlying urban areas.

THE " RAT 5 " STRAIN.

This strain was initiated by the inoculation into one guinea-pig and one wild rat of a suspension of the brain, spleen and testis of a rat that had been trapped in an endemic area of rural typhus. Febrile reaction and scrotal swelling resulted in the guinea-pig, but the rat showed no reaction during 10 days' observation. On the 10th day two similar suspensions were made, one from the rat's and another from the guinea-pig's organs, and each was inoculated by the intra-peritoneal route into a group of guinea-pigs. Febrile reaction and scrotal swelling occurred in animals of each group. The guinea-pigs of the early generations of passage were fed on a diet deficient in vitamin A ; later this diet was found to be no longer necessary (as with the " Rat 4 " strain described above). The strain was maintained in the guinea-pig for two years without loss of virulence, and then discarded. The majority of guinea-pigs survived the febrile reaction.

The Infection in the Guinea-pig.

The nature of the passage virus and the route of inoculation used were similar to those noted above in the case of the " Rat 4 " strain. The incidence of fever and scrotal reaction, the post-mortem signs and the findings in smears from spleen and testis were also similar to those noted above in the case of the " Rat 4 " strain.

The Infection in the Rabbit.

From time to time rabbits were inoculated by the intra-peritoneal or intra-ocular route with passage virus from guinea-pigs, in order to demonstrate by the result of the Weil-Felix reaction the continued typhus-like nature of the infection. Of 42 rabbits inoculated by the intra-peritoneal route, the sera of 27 gave a positive reaction ; titres were high, 7 exceeding 1/500, 2 being 1/1000 and 1/1925 respectively. In the great majority of these rabbits the agglutination was of the OXK type only ; but in a considerable minority the sera reacted only with the OX19 type (for discussion of this unexpected result see p. 223).

Three rabbits were inoculated by the intra-ocular route on separate occasions with tunica exudate from 2 guinea-pigs and heart-blood from 1 guinea-pig, obtained at the height of reaction. An ocular reaction resulted in all three that in incubation period and intensity and evolution of signs was identical with that seen in similar infections with the viruses of rural typhus

and the tsutsugamushi disease of human origin. Similarly, also, the rabbits recovered, and were then immune to reinfection with the homologous virus for a period of at least nine months. Moreover, by the methods described in an earlier paper, *Rickettsia* could be demonstrated with ease in scrapings from the endothelium covering Descemet's membrane in the reacting eye; in morphology, distribution and staining characteristics they were identical with those demonstrable in the endothelial cells of the tunica exudate from reacting guinea-pigs of this same strain and of the "Rat 4" strain, and in infected material from guinea-pig and rabbit strains of rural typhus and the tsutsugamushi disease of human origin, *i. e.* with *Rickettsia orientalis*.

By passage of infected aqueous humour a strain was maintained in the rabbit without difficulty for 31 generations. Sera from 14 passage rabbits were tested for the Weil-Felix reaction; 7 were positive, titres exceeding 1/500 in 2. Agglutination was invariably of the OXK type only.

It will be seen that these two rat strains had many characteristics in common, viz. the similarity of the incubation period, febrile and scrotal reactions, post-mortem findings, and the *Rickettsia* demonstrable in infected material. They differed, however, in two characteristics, viz. the inability of the "Rat 4" virus to survive in rabbits, even to the second generation; and, in the case of the "Rat 5" strain, the development of agglutinins of the OX19 type in a considerable minority of positive sera, in contrast to the pure OXK type of agglutinins that developed in the majority of positive sera of this strain and in all those of the "Rat 4" strain.

As maintenance of these two strains proceeded it was discovered that there was in each case a concomitant infection with the *Spirillum morsus muris*. Low secondary febrile reactions and progressive enfeeblement of the guinea-pigs were common; and the spirillum could be demonstrated in the blood during the third week and, less often, in the tunica exudate. The presence of this additional infection may have contributed to the comparative ease with which guinea-pigs could be infected, which was in marked contrast with the authors' experiences in experimental infections with strains of rural typhus and the tsutsugamushi disease of human origin (see Parts I and II). Suzuki (1934) found the same concomitant in a series of wild rats caught in Hamburg, and shown by him to be infected with typhus virus of the X19 type.

Difficulties that had impeded early attempts to carry out cross-immunity tests between these two rat strains in guinea-pigs were thus explained, and the tests were abandoned. Use of rabbits, however, was successful. The passage rabbits of the "Rat 5" strain, maintained by the intra-ocular inoculation of virus, showed no generalized ill-effects during or after the ocular reaction, and could be used as immunes for cross-immunity experiments, in some cases as long as nine months after their initial inoculation. A detailed account of cross-immunity tests is deferred until a later paper. It may be stated briefly here that rabbits convalescent after infection with a strain of the tsutsugamushi disease or any of four strains of rural typhus (all these five being of human origin) proved to be immune to reinfection with the virus of the "Rat 5" strain; and conversely. In the case of the "Seerangayee" strain of rural typhus (also of human origin), transferred for the purpose from guinea-pigs

to rabbits by the intra-ocular inoculation of infected heart-blood, 3 convalescent rabbits of the "Rat 5" strain showed complete immunity to reinfection with the virus of the "Seerangayee" strain, and 3 others a partial immunity.

Marcandier and Pirot (1933) and Chuiton and Moreau (1933) have compared the experimental infections in guinea-pigs due to a strain of murine typhus and to one of sodoku. It is evident from their findings that differing features of febrile and scrotal reactions cannot always be regarded as safe criteria of differentiation between the two conditions. In our experiments with the viruses of the "Rat 4" strain and the "Rat 5" strains, however, four additional criteria were available, viz. (1) the finding of abundant intracellular *Rickettsia*, indistinguishable from *R. orientalis*, in material from infected guinea-pigs and rabbits; (2) the development in the sera of infected rabbits of agglutinins ("O" type) for strains of *Proteus X* to titres that, for the rabbit, must be considered to be significant; (3) the successful experimental infection of rabbits with the virus of one strain by the intra-ocular route, and the entire correspondence of these infections with those obtained in experimental rural typhus and tsutsugamushi; (4) the demonstration of a cross-immunity, complete in five strains and partial in a sixth strain, between strains of rural typhus and tsutsugamushi of human origin on the one hand and the "Rat 5" strain on the other hand.

In Part I we drew attention to the distinction made by Nicolle (1933) between the virus of Old World louse-borne typhus and the virus of murine typhus; that the former could not be maintained by him beyond the 13th generation in three separate series of white rats, while the latter virus could be maintained experimentally in the white rat indefinitely. We showed that the virus of a strain of rural typhus of human origin could be so maintained for 21 generations without loss of virulence, and presumably, therefore, indefinitely. The findings reported in this present paper, together with those summarized in the introduction, can leave no doubt that the rat is a reservoir of the virus of rural typhus and the tsutsugamushi disease, *i. e.* that these viruses are murine in type.

SUMMARY.

(1) Two strains of tropical typhus have been isolated from wild rats trapped in endemic areas.

(2) Both were isolated and maintained in guinea-pigs.

(3) One could be isolated in rabbits by the intra-ocular inoculation of virus, and maintained thus indefinitely. The features of this infection appeared to be identical with corresponding infections obtained with the viruses of rural typhus and the tsutsugamushi disease of human origin. Repeated attempts to isolate the other rat strain in rabbits in the same manner failed.

(4) *Rickettsia* were found with ease and in abundance in infected material from guinea-pigs infected with either strain, and from rabbits infected with the one strain.

(5) The sera of rabbits infected with the two rat strains gave positive Weil-Felix reactions of significant titre.

(6) Cross-immunity tests in rabbits between one rat strain and six strains

of human origin of rural typhus and tsutsugamushi showed a cross-immunity to exist, complete in five strains and partial in the sixth strain.

(7) A concomitant infection with sodoku was present in the guinea-pigs of both strains ; although this may have modified the clinical signs, the infection by a typhus virus could be determined by four decisive criteria.

(8) The conclusion is drawn that the murine origin of the virus of the rural typhus-tsutsugamushi group of diseases is now firmly established.

REFERENCES.

ANIGSTEIN, L.—(1933) " Studies on Tropical Typhus," *Stud. Inst. med. Res. F.M.S.*
CHUITON, F. LE, AND MOREAU, M.—(1933) *C. R. Soc. Biol.*, Paris, **112** : 1380.
FLETCHER, W., LESSLAR, J. E., AND LEWTHWAITE, R.—(1928) *Trans. Roy. Soc. trop. Med. Hyg.*, **22** : 161.
GATER, B. A. R.—(1930) *Trans. Far Eastern Ass. trop. Med.*
LEWTHWAITE, R., AND SAVOOR, S. R.—(1936) *Brit. J. exp. Path.*, **17**, 1.
MARCANDIER, A., AND PIROT, R.—(1933) *Bull. Soc. Path. exot.*, **26**, 32.
NICOLLE, CH.—(1933) *Arch. Inst. Pasteur*, Tunis, **21**, 349.
SUZUKI, K.—(1934) *Zbl. Bakt.*, I Abt. Orig., **131**, 236.

THE TYPHUS GROUP OF DISEASES IN MALAYA. PART V: THE WEIL-FELIX REACTION IN LABORATORY ANIMALS.

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FLETCHER and Lesslar (1926) recognized the existence in Malaya of two forms of tropical typhus, the rural form and the urban form (referred to in this paper for brevity as rural typhus and urban typhus respectively). Though much alike in their clinical features they were shown to be distinct diseases, distinguished one from another by their epidemiology and by the Weil-Felix reaction ; to these differential criteria others have since been added. In the case of rural typhus, sera from convalescent patients agglutinated the OXK type of *Proteus X* strains, never the OX19 type ; in the case of urban typhus, corresponding sera agglutinated the OX19 type, never the OXK type. Further, these authors emphasized the necessity of making repeated examinations in every case, the evidence of a waxing and waning titre constituting proof of active infection.

In the case of the tsutsugamushi disease, which, chiefly on clinical grounds, has hitherto been regarded as the third member of the typhus group to occur in Malaya, Fletcher and Field (1927), and Fletcher, Lesslar and Lewthwaite

(1929), describing the few cases that had then been observed, record agglutination of the OXK type, but only to titres much lower than had been obtained in rural typhus. In a larger series of cases of tsutsugamushi subsequently observed by the present authors the occurrence of the OXK type of agglutination was amply confirmed, being invariable where repeated samples of serum were available during convalescence; moreover, the titres were equally as high as those obtained in rural typhus, readings higher than 1/2000 being not infrequent. The findings of Felix and Rhodes (1931), who first showed this constant agglutination to occur in cases from Japan and Sumatra, were thus confirmed. Similar serological findings in the case of both rural typhus and the tsutsugamushi disease have been recorded in Sumatra by Wolff (1931).

In experimental rural typhus it was shown by one of us (Lewthwaite, 1930) and by Anigstein (1933) that the intra-peritoneal inoculation of virus into rabbits resulted in the development of agglutinins to the OXK type; agglutination was, however, low in titre and uncertain in incidence. Thus Anigstein reported maximum titres of 1/125 to 1/250; and noted, as Felix (1933) had first described, the dependence of the degree of agglutination on the varying susceptibility of individual rabbits, and on the origin and quantity of the virus inoculated. In the short series previously reported by one of us 11 rabbits had been inoculated intra-peritoneally with blood drawn from patients at the height of fever; in 8 the Weil-Felix reaction remained negative; in 3 it was positive, but the titres did not exceed 1/125.

METHODS AND CRITERIA USED IN THE PRESENT INVESTIGATION.

The present report comprises results obtained by inoculation of rabbits and monkeys with the respective viruses of rural typhus, urban typhus and the tsutsugamushi disease; a large series of rabbits has been thus inoculated by the intra-peritoneal and intra-ocular routes, several monkeys and gibbons by the intra-dermal route. The inocula were mostly infected material from guinea-pigs and rabbits in which strains of these diseases had been isolated from human sources and were maintained by serial passages. (Brief summaries of these serum reactions have been given in Parts I–III of this series of papers (1936).) The Weil-Felix reactions following infection with the two strains of virus of tropical typhus of rat origin (see Part IV) are included also.

All agglutination tests were made with both living and alcohol-treated suspensions of *Proteus X* strains. The OXK strain used was one supplied by the courtesy of Dr. Felix; the OX19 strain used was one supplied by the courtesy of Prof. W. A. Young, from the laboratory of the Department of Bacteriology, King Edward VII College of Medicine, Singapore. (Details of the history and characteristics of these two strains are to be found in the Annual Reports of this Institute for the years 1931–3.)

In all cases blood was drawn from the animal before inoculation, and subsequent to inoculation at intervals of 5 days from the 10th day onwards. In a few early cases, that formed a small minority, observation was continued for 35 days only. Felix (1933) has stressed the occasional occurrence of an extremely late development of agglutinins for *Proteus X*19 in rabbits infected with the virus of louse-borne typhus, and in a prior personal communication

very kindly brought this fact to our notice early in the progress of this investigation. Thereafter the period of observation of inoculated rabbits was prolonged to 60 days. In many instances significant agglutination of the OXK type was attained only after the 40th day, and, in one instance, not until the 54th day of observation.

As to the standards by which the Weil-Felix reactions were adjudged positive on the one hand and inconclusive or negative on the other hand, two were regarded as of equal importance, viz. the height of titre attained, and the progressive rise and decline in degree of titre. These two criteria are complementary, as Felix (1930) has emphasized, and were almost invariably conclusive.

The titres of agglutination given by the serum before inoculation of the rabbit were either zero or very low in the case of both the OXK type and the X19 type, being almost invariably zero in the latter type. The following table summarizes the findings:

Agglutinin Titres of the Sera of 310 *Normal Rabbits.*

Range of titre of agglutinins.	Number of sera that agglutinated *Proteus X* strains.			
	OXK.		OX19.	
	Number.	p.c.	Number.	p.c.
0–25	276	89	308	99·4
25–50	29	9·4	2	0·6
50–75	5	1·6	0	0

In the case of the OXK type, a titre below 1/50 was therefore regarded as negative; a titre between 1/50 and 1/75 as inconclusive unless the form of the curve of successive titres showed the waxing and waning features characteristic of active infection; and a titre greater than 1/75 as positive. In the case of the OX19 type the interpretation was simpler; a titre below 1/25 was always regarded as negative, one lying between 1/25 and 1/50 looked upon with suspicion, and one exceeding 1/50 as positive. As with the OXK type, the waxing and waning curve was always as essential to acceptance of a reaction as being positive.

The initial titres and the standards applied in the case of the 2 gibbons and 5 monkeys used for experiment were the same as for the rabbit.

THE WEIL-FELIX REACTION IN THE RABBIT.

Rural Typhus.

Inoculated by the intra-peritoneal route.—Sera from 2 rabbits thus inoculated with 5 c.c. of infected blood from two patients were examined; both were negative. Sera from 27 rabbits inoculated with passage virus (aqueous humour or guinea-pig tissues) from 4 strains of rural typhus were tested. 18 proved to be positive; in 5 the maximum titres lay between 1/50–1/75; in 6 between 1/75–1/100; in 5 between 1/100–1/200; and in 2 between 1/200–1/300.

Inoculated by the intra-ocular route.—Sera from 2 rabbits inoculated by this route with 0·1 c.c. of blood from a patient suffering from rural typhus were

tested ; both were positive, maximum titres of 1/300 and 1/380 being obtained on the 33rd day after inoculation. Sera from 24 rabbits thus inoculated with passage virus (aqueous humour) from 4 strains of rural typhus were tested. Ten proved to be positive ; in 5 the maximum titre lay between 1/50–1/75 ; in 2 between 1/75–1/100 ; in 2 between 1/100–1/200 ; in 1 between 1/200–1/300. Maximum titres were attained in most cases towards the end of the 2nd or during the 3rd week after inoculation, in a few cases during the 4th and 5th weeks, and in 3 cases not until the 9th week. Twelve rabbits inoculated by the intra-ocular route reacted with ocular signs, yet their sera failed to develop agglutinins to significant titres ; in none, however, did the converse occur.

Agglutination was only of the OXK type.

Tsutsugamushi.

Inoculated by the intra-peritoneal route.—Only one such inoculation was made, 4 c.c. of infected human blood being inoculated into a rabbit. The serum gave a positive reaction, a maximum titre of 1/110 being reached on the 28th day.

Inoculated by the intra-ocular route.—Six sera from rabbits inoculated thus with infected blood from two patients were tested : 2 were positive, maximum titres lying between 1/50–1/75. Sera from 57 rabbits inoculated thus with passage virus (aqueous humour) were tested : 34 were positive ; in 5 the maximum titre lay between 1/50–1/75 ; in 8 between 1/75–1/100 ; in 11 between 1/100–1/200 ; in 3 between 1/200–1/300 ; in 4 between 1/300–1/400 ; in 2 between 1/400–1/500 ; in 1 it was 1/1925. The particular week after inoculation during which the maximum titres were reached varied evenly from the 2nd to the 7th in 31 of the above 34 animals ; in 3 it was the 8th, and in 1 the 9th week. Nineteen rabbits gave a marked ocular reaction, yet their sera gave a negative Weil-Felix reaction ; the converse occurred in 1 case only.

Agglutination was always of the OXK type only.

Urban Typhus.

Inoculated by the intra-peritoneal route.—One rabbit was thus inoculated with infected human blood. The serum gave agglutination to a titre of 1/38—sufficiently high to be significant, since it was of the OX19 type and the waxing and waning features were present. Seven rabbits were thus inoculated with passage virus (guinea-pig tissues) ; the sera from 3 of them were positive, the maximum titre lying between 1/25–1/50 in 2, and 1/50–1/75 in 1.

Inoculated by the intra-ocular route.—Sera were tested from 4 rabbits thus inoculated with infected blood from one patient ; in all the Weil-Felix reaction was negative. Sera were tested from 58 rabbits infected thus with passage virus, that consisted variously of blood or tunica exudate from infected guinea-pigs, infected aqueous humour, or suspensions of crushed fleas infected with the laboratory strain of urban typhus. Sera from the 3 rabbits inoculated with the flea suspensions were negative, although intense reactions in control

guinea-pigs inoculated intra-peritoneally proved the virulence of the inoculum. Sera from 30 of the other 55 rabbits gave a positive Weil-Felix reaction; in 7 the maximum titre lay between 1/25–1/50; in 10 between 1/50–1/100; in 3 between 1/100–1/200; in 2 between 1/200–1/300; in 3 between 1/300–1/400; in 2 between 1/400–1/500; in 1 between 1/700–1/800; while two titres were as high as 1/1700 and 1/1925 respectively. In 4 rabbits the sera gave a positive Weil-Felix reaction, yet ocular signs were absent; conversely, in 6 rabbits the sera gave a negative Weil-Felix reaction, yet ocular signs were present. It is noteworthy that an inoculum of tunica exudate was no more effective than one of heart-blood or aqueous humour.

In this series of 34 positive sera maximum titres were attained in the large majority during the 3rd week, in a few during the 2nd or 4th week, in 1 only as late as the 5th week. The two highest titres, given above, were recorded on the 26th and 17th days after inoculation respectively.

Agglutination was invariably of the OX19 type only.

Strains of Tropical Typhus Isolated from Wild Rats.

As described in Part IV, 2 strains were recovered from 2 wild rats; both were isolated and maintained in guinea-pigs, and one could be transferred to rabbits by the intra-ocular injection of virus.

The "Rat 4" Strain.

Inoculated by the intra-peritoneal route.—Sera from 11 rabbits inoculated thus with passage virus (guinea-pig tissues) were tested: 9 proved to be positive; in 1 the maximum titre lay between 1/75–1/100; in 1 between 1/100–1/200; in 3 between 1/200–1/300; in 1 between 1/300–1/400; in 1 between 1/400–1/500; in 1 between 1/500–1/600; in 1 between 1/600–1/700.

Inoculated by the intra-ocular route.—In 13 instances attempts were made to isolate and maintain this strain in the rabbit by the intra-ocular injection of virus from infected guinea-pigs. All attempts failed. In none did any ocular reaction occur. Sera from 12 of the rabbits were submitted for the Weil-Felix reaction: 8 were positive; in 5 the maximum titre lay between 1/100–1/200; in 1 between 1/200–1/300; in 1 between 1/700–1/800; in 1 it was 1/1700. Maximum titres were reached during the 4th week in most cases, the 3rd in a few, the 2nd, 5th and 6th in 1 case each.

Agglutination by sera of this strain was invariably of the OXK type only.

The "Rat 5" Strain.

Inoculated by the intra-peritoneal route.—Sera from 42 rabbits inoculated thus with passage virus (guinea-pig tissues) were tested, and 27 were positive; 18 agglutinated only the OXK type, 8 agglutinated only the OX19 type, while 1 agglutinated both types. (The apparent inconsistency of these results will be discussed more fully below.) In the 18 OXK type sera the maximum titre lay between 1/50–1/75 in 3; 1/75–1/100 in 3; 1/100–1/200 in 3; 1/200–1/300 in 2; 1/300–1/400 in 1; 1/500–1/600 in 1; 1/600–1/700 in 2; 1/900–

1/1000 in 2 ; and was 1/1925 in 1. In the 8 OX19 type sera the maximum titre lay between 1/25–1/50 in 1 ; 1/50–1/75 in 1 ; 1/75–1/100 in 2 ; 1/100–1/200 in 2 ; 1/200–1/300 in 1 ; and was 1/825 in 1. The serum that gave dual agglutination gave maximum titres of 1/170 OXK on the 11th day, and 1/96 OX19 on the 14th day after inoculation.

Inoculated by the intra-ocular route.—Sera from 14 rabbits thus inoculated with passage virus (tunica exudate or heart-blood of a guinea-pig) were tested, and 7 were positive, all agglutinating the OXK type only. The maximum titre lay between 1/100–1/200 in 3 ; 1/200–1/300 in 2 ; 1/500–1/600 in 1 ; 1/600–1/700 in 1. Six reacted with ocular signs, yet their sera gave a negative Weil-Felix reaction ; on the other hand, 4 rabbits failed to react with ocular signs, yet their sera gave a positive Weil-Felix reaction. The particular week during which maximum titres were reached was, in the case of sera giving only OXK type agglutination, the 3rd and 4th week in the majority ; in a few it was the 2nd, 5th, 6th and 7th ; in 5 it was as late as the 9th–11th week. In the case of the sera agglutinating only the OX19 type, it was the 2nd week in 6 cases, and the 3rd week in 2 cases.

General Observations.

In reviewing the above results of experimental infection in the rabbit, certain general observations appear noteworthy.

It has been observed repeatedly that the intra-ocular inoculation into rabbits of 0·1 c.c. of virus, whether infected blood or tunica exudate, has resulted in the development of agglutinins to titres as high as, and sometimes much higher than, those resulting from inoculation of much larger quantities, viz. 6–10 c.c., of the same inoculum by the intra-peritoneal route. Agglutination of the OX19 type develops to significant titres and reaches its maximum early. Agglutination of the OXK type develops more slowly, though individual strains vary somewhat in this respect ; the maximum titres were not reached until the 9th week in 8 rabbits, and the 11th week in 1 rabbit. This distinction is well shown by the results of the Weil-Felix reactions of rabbits of the " Rat 5 " strain, for here inoculation of the virus, in most cases the same inoculum (tunica exudate), of the one strain caused the development of agglutinins of either type. This early appearance of OX19 agglutinins accords with the findings of Weil and Felix (1921) in rabbits infected by the intra-peritoneal route with guinea-pig tissue virus of louse-borne typhus of East European origin.

In the sera of rabbits inoculated with the virus of rural typhus, urban typhus, or the tsutsugamushi disease, agglutinins for OXK or OX19 develop in no more than 55 p.c., whether the route of inoculation be intra-peritoneal or intra-ocular. Herein lies another difference between these strains of virus of endemic typhus and those of the louse-borne epidemic variety ; for in the latter Weil and Felix (1921) have shown that agglutinins develop in almost 100 p.c. of infected rabbits.

Following infection of rabbits by the intra-ocular method, an ocular reaction is more frequent than is a positive Weil-Felix reaction, in some strains much more so than in others.

THE WEIL-FELIX REACTION IN THE MONKEY.

Rural Typhus.

One gibbon and 3 macacus monkeys were inoculated on separate occasions by the intradermal route, at multiple sites, with virus that consisted of infected material from guinea-pigs or rabbits reacting to the inoculation of a rural typhus strain (*vide* Part I).

In the gibbon.—This was inoculated with scrapings of the endothelium covering Descemet's membrane in the reacting eye of 2 rabbits of the " Seerangayee " strain of rural typhus, the strain having been transferred from guinea-pigs to rabbits for the purpose of these experiments by the intra-ocular inoculation of infected heart-blood. Agglutination of the OXK type was obtained, the maximum titre of 1/170 being reached on the 21st day after inoculation. There occurred also a concomitant agglutination of the OX19 type, the maximum titre of 1/1925 being reached on the 15th day. This dual agglutination will be discussed below.

In macacus monkeys.—The inoculum for 1 of these was the same as for the gibbon, but from another rabbit; that for the other 2 was peritoneal fluid and fibrin from the surface of the spleen of a reacting guinea-pig of the " Seerangayee " strain of rural typhus. The sera from all three developed only agglutinins for the OXK strain, maximum titres of 1/141 in 1 and 1/125 in 2 being reached respectively on the 14th, 10th and 28th days after inoculation.

The Tsutsugamushi Disease.

One gibbon and 1 macacus monkey were inoculated intra-dermally at multiple sites with scrapings of the endothelium covering Descemet's membrane in the reacting eye of passage rabbits of a strain of the tsutsugamushi disease. Both reacted fully (see Part II). In both the sera gave a positive Weil-Felix reaction, the OXK strain alone being agglutinated; a maximum titre of 1/3850 was reached on the 28th day after inoculation in the gibbon, and 1/385 on the 20th day in the macacus.

Urban Typhus.

One gibbon and 2 macacus monkeys were inoculated intradermally at multiple sites with tunica exudate from reacting guinea-pigs of a strain of urban typhus; and all reacted with fever, leucopenia, and positive agglutination reactions. The gibbon was the same animal that had been used a few months previously for intradermal inoculation with the virus of tsutsugamushi (*vide supra*), the complete lack of cross-immunity between the rural typhus-tsutsugamushi group of diseases and urban typhus, known from guinea-pig and rabbit experiments, permitting this procedure. Following inoculation with the virus of urban typhus agglutinins for OXK that had fallen to zero showed no significant rise; but agglutinins for OX19 rose from zero, to reach a maximum titre of 1/220 on the 20th day after inoculation.

One of the 2 macacus monkeys had also been inoculated intradermally with the virus of the tsutsugamushi disease three months previously. Here,

also, following reinoculation, there was no significant rise of OXK type agglutinins; but agglutinins for OX19 rose from zero, to reach a maximum titre of 1/110 on the 19th day. The other macacus had not previously been inoculated. Agglutinins for OX19 rose from zero, to reach a maximum titre of 1/96 on the 15th day after inoculation; no significant rise of OXK agglutinins occurred.

THE OCCURRENCE OF CHANGE IN TYPE OF ANTIBODY RESPONSE.

We have more than once obtained serological results that were at variance with those that usually followed inoculation of a particular virus into a rabbit or monkey. In some instances inoculation of a virus strain that had previously stimulated only OXK type agglutination would cause agglutination of both OXK and OX19 types; in others such a strain would give OX19 type agglutination alone.

Anigstein (1933) carried out Weil-Felix reactions on the sera of rabbits inoculated with the virus (human or passage) of rural typhus. He reports: "It is of interest to note that the virus of K type did not always provoke homologous agglutinins for the XK strain, as against some rabbits' sera the only reacting strain was OX19; certain sera agglutinated both strains while others responded to the inoculation of the K virus by X19 agglutinins only." The same worker describes the following experience:

> Blood from a human case of rural (*i. e.* K type) typhus, after 48 hours' incubation in Hottinger broth, was inoculated into a guinea-pig. The virus was passed in series through this and another guinea-pig into a rat. Both guinea-pigs reacted with fever; the rat developed marked scrotal swelling, and *Rickettsia* were abundant in the tunica vaginalis; also its serum agglutinated only the OX19 type, to a titre of 1/250. Passage was continued by the inoculation of tunica exudate from this rat into another guinea-pig; this reacted with fever and scrotal swelling, and *Rickettsia* were demonstrable in the tunica vaginalis. The brain of this guinea-pig was inoculated into a human volunteer, who developed a mild fever for 10 days, and gave a Weil-Felix reaction of 1/500 HX19 and 1/125 OX19, there being no agglutination of XK; one week later the titre for HX19 had risen to 1/1000, but there was still no agglutination of XK.

With regard to these serological findings, Anigstein observed that they indicate "that a distinct serological change in the antigenic properties of the virus took place by animal passages, namely, the transformation of the original K type into the W (*i. e.* X19) form".

With regard to the above experience, mention is not made of the pre-inoculation titre of the serum of the rat; and it is necessary to bear in mind the warning of Felix (1933) that "the agglutination reactions obtained with *Proteus X* strains and the serum of wild and tame rats ought to be interpreted with the utmost caution". Nevertheless the development of scrotal swelling and the finding of *Rickettsia* in the rat point strongly to an active infection of this rat with a typhus virus.

Observations on Two Human Cases of Tropical Typhus.

We have seen two human cases of typhus-like fever, both from the same town, in which the Weil-Felix reactions showed significant agglutination of both OXK and OX19 types. The first patient, "T. M.", lived in a rat-infested house, and, some two weeks before onset of illness, had killed and

buried several rats. The clinical syndrome was that of tropical typhus (the two serological varieties of which cannot be differentiated on clinical criteria). The Widal reaction was repeatedly negative. The Weil-Felix reactions were as follows :

Patient " T. M." : Agglutination Titres.

Day of illness.	Living cultures. OXK.	OX19.
9	30	85
11	0	300
14	85	440
19	680	500
24	680	385
30	440	300
41	300	150
55	150	150

The second patient, " M. V." was seen only during convalescence, and we were unable to obtain more than three samples of sera. The clinical syndrome had been that of tropical typhus. The Weil-Felix reactions were as follows :

Patient " M. V." : Agglutination Titres.

Day after onset.	Living cultures. OXK.	OX19.
11	170	85
17	340	170
35	125	96

In regard to these two cases, a remotely possible suggestion might be that the infection observed was the second of two consecutive typhus-like infections, and that it had re-stimulated residual agglutinins of the other type, contrary to the view of Felix (1929), who maintains that the " O " type of agglutinins is not liable to non-specific re-stimulation. There was no history of a previous infection, although, of course, this might have been " inapparente " in form. It is also possible that the attack was due to both viruses having been inoculated together, since the rat is a reservoir of both serological varieties of tropical typhus. A third possibility is that the infecting virus was single but of unusual antigenic structure, by reason of which it evoked a serological response to both types of *Proteus X* strains. Pijper and Dau (1935) record a similar case in a series of human sera from cases of a mild typhus-like disease that broke out in Pretoria, and which these workers considered to be due to a virus from rats, and to be conveyed to man by means of rat fleas. The serum agglutinated both OXK and OX19 types to a titre of 1/1280.

Observations on the " Rat 5 " Strain of Virus.

During maintenance of the " Rat 5 " strain, as noted above, 42 rabbits were inoculated intra-peritoneally with passage virus (tunica exudate or brain) : the Weil-Felix reaction was positive in 27 sera ; of these, 18 agglutinated only

the OXK type, 8 only the OX19 type, and 1 both types. Six of these 8 were 3 pairs that were inoculated on three separate occasions during November and December, 1931, when the strain was first isolated. The rabbit of which the serum gave a dual agglutination was one of a pair; both were inoculated from the same syringe with the same inoculum of diluted tunica exudate from a reacting guinea-pig. The Weil-Felix reactions are given below:

Agglutination Titres.

Day after inoculation.	Rabbit 134. Living cultures.		Rabbit 135. Living cultures.	
	XK.	X19.	XK.	X19.
0	56	0	0	0
10	150	17	30	770
11	170	50	0	825
14	96	96	50	560
19	0	0	0	190
24	85	17	0	0

Agglutinins were purely "O" in type.

It is seen that the serum of Rabbit 134 shows dual agglutination; that of Rabbit 135 shows X19 agglutination only, since the low XK agglutinins cannot be regarded as significant. On July 17th, 1932, a rabbit (No. 437) was inoculated by the intra-ocular route with diluted tunica exudate (0·1 c.c.) from a reacting guinea-pig of the "Rat 5" strain. Seven samples of sera were examined at intervals between the 12th and the 50th days after inoculation. Agglutinins for OXK rose from a pre-inoculation titre of 1/38, to reach a maximum titre of 1/220 on the 23rd day; the typical waxing and waning features were present; no agglutination of the OX19 type occurred. On July 23rd, 1932, *i. e.* 6 days later than the above inoculation, another rabbit (No. 445) was inoculated by the intra-peritoneal route with diluted tunica exudate (2 c.c.) from another reacting guinea-pig of the "Rat 5" strain. Eight samples of sera were examined at intervals between the 11th and 54th days after inoculation. Agglutinins for the OX19 type rose from a pre-inoculation titre of zero to reach a maximum titre of 1/220 on the 11th day. The early appearance of significant agglutinins, typical of OX19 type of agglutination, was present; there was typical waxing and waning of titres; agglutination of the OXK strain was zero before inoculation, and remained zero throughout. Three months later another rabbit was inoculated by the intra-peritoneal route with diluted tunica exudate from a reacting guinea-pig of the "Rat 5" strain; the serum developed agglutinins for OXK to high titres that reached a maximum of 1/1925 in the 58th day; OX19 agglutination was *nil*. During the course of the next 12 months (at the end of which the strain was discarded) 23 rabbits that had been inoculated on separate occasions, the majority by the intra-peritoneal route, a few by the intra-ocular route, usually with tunica exudate, occasionally with heart-blood or brain, gave positive Weil-Felix reactions of the OXK type only; no case of OX19 agglutination again occurred.

Observations on the "Seerangayee" Strain.

The following experience has already been referred to briefly in Part I of this series. In November, 1934, the authors had occasion to inoculate a gibbon (No. 2), not previously used for experiment, with virus from the "Seerangayee" strain of rural typhus. To provide the inoculum, the strain was established in rabbits by the intra-ocular injection of infected heart-blood from guinea-pigs of this strain; and, from one rabbit of the 2nd generation and one of the 3rd, aqueous humour and scrapings of the endothelium covering Descemet's membrane were secured at the height of ocular reaction. The gibbon underwent a severe infection characteristic of experimental rural typhus, and marked by fever, leucopenia, necrotic dermal lesions and a bubo (as described and illustrated in Part I). On the 13th day after defervescence and 26th day after inoculation, it died from a cellulitis that accompanied a flaring-up of an old skin disease. Samples of sera were obtained, one before and five after inoculation; the results of the Weil-Felix reactions were as follows:

Sera from Gibbon No. 2: Agglutination Titres.

Days after inoculation.	Living cultures. OXK.	OX19.
0	22	25
8	22	22
12	17	340
15	50	1925
21	170	1000
25	50	500

It is seen that although significant agglutination of the OXK type occurred, to a maximum titre of 1/170 on the 21st day, there also occurred a predominant agglutination of the OX19 type, to a maximum titre of 1/1925 on the 15th day—an event entirely unexpected, for since the isolation of this strain of virus three years previously it had behaved consistently as an XK type and never as an X19 type virus (classification according to Felix and Heatley-Spencer, 1935). Since, however, the serum of the patient "Seerangayee", from whose blood the strain was isolated, gave a dual agglutination, the origin and characteristics of the strain will be briefly described. The patient worked on an estate on which rural typhus had been endemic for many years. The clinical syndrome was that of tropical typhus. The serum agglutinations were as follows:

Sera from Patient "Seerangayee": Agglutination Titres.

Day after onset of illness.	Living cultures. OXK.	OX19.
11	1100	1900
14	770	680
21	1900	110
28	5000	190
31	3800	110

It is seen that on the 11th day of illness agglutination was of both the OXK and OX19 types, to significant titres ; and that subsequently the OXK type agglutinins rose sharply, while the OX19 type declined equally sharply, *i. e.* the waxing and waning features of the titres were characteristic in each type of those met with in rural and urban typhus respectively. There was no history of a previous typhus-like infection ; a series of vaccination experiments amongst the labour force of this estate had ended several months before the patient joined the estate.

Two guinea-pigs that had been fed on a diet deficient in vitamin A for 10 days were inoculated intra-peritoneally with 3 c.c. of blood, drawn from the patient early in the course of the fever. Both reacted with fever, and from one of them a strain was established and maintained in guinea-pigs, and still survives with unabated virulence. The characteristics of this strain in the guinea-pig were described in Part I ; they differ from those of urban typhus in the guinea-pig in that scrotal swelling is absent, 90 p.c. of guinea-pigs die, ascites is found at post-mortem examination, and the morphology of the causal *Rickettsia* is that of *R. orientalis* and not that of *R. prowazeki* ; moreover a large series of cross-immunity tests in the guinea-pig has shown complete lack of cross-immunity between this strain and an urban strain of virus of the X19 type. The " Seerangayee " strain can be transferred with ease to rabbits by the intra-ocular inoculation of heart-blood from a reacting guinea-pig ; further, it can be maintained in them indefinitely, quite contrary to our experiences with the OX19 type of virus. The characteristics of the infection in the rabbit, viz. ocular reaction, *Rickettsial* findings and serology, have been identical with those of four other strains of rural typhus isolated in the rabbit from patients whose sera gave high agglutination of the OXK type alone. Cross-protection tests carried out in the rabbit between the " Seerangayee " strain and 3 strains of the type XK showed a complete cross-immunity to exist ; on the other hand, tests between this strain and an urban typhus strain of the X19 type showed entire lack of cross-immunity in the rabbits. In the monkey the intradermal inoculation of this virus gave the same typical necrotic ulcer at the site of inoculation as did the virus of another XK type of virus ; an OX19 type of virus, on the other hand, gave no trace of dermal lesion at the site of intradermal inoculation.

Furthermore, we have recently shown that under laboratory conditions the rat flea (*X. cheopis*) can acquire and transmit the virus of urban typhus (an X19 type of virus), intense infections resulting. In a collateral investigation in which the virus of the " Seerangayee " strain was used, no evidence that the flea could transmit or even acquire the virus was obtained. More recently, by a modified method, one of us (S. R. S.) has repeated this experiment, and obtained some evidence that may be interpreted as indicating that the rat flea has a feeble power to transmit this virus. But the few reactions obtained were very mild in character, and suggestive only of the XK type of infection, being totally different from those due to transmission by the rat flea of the X19 type of virus. Yet, if the " Seerangayee " strain were a mixture of an XK and an X19 type of virus, it is reasonable to suppose that within the flea it would have multiplied, and been demonstrable in the white rat or guinea-pig by the inoculation of crushed fleas or the

feeding of the fleas precisely as was the laboratory strain of the X19 type of virus.

Again, the serological results obtained from time to time in rabbits infected with the " Seerangayee " strain since its initiation showed no trace of agglutination of the X19 type. Thus, in the course of three years, 37 rabbits have been inoculated at various times by the intra-peritoneal or the intra-ocular route ; the sera of 24 gave a positive Weil-Felix reaction. Agglutinins were always for OXK, never for OX19.

It is thus seen that the " Seerangayee " strain had hitherto behaved as a true XK type in the guinea-pig, rabbit and monkey by every available criterion—clinical, serological, immunological, epidemiological (under laboratory conditions)—and by the morphology of the causal *Rickettsia*.

To elucidate the apparently contradictory serological results in the gibbon the authors studied a daughter strain that had been isolated from the gibbon, and also inoculated more monkeys with the virus of the parent strain. At the height of the febrile reaction of the gibbon, blood had been drawn from the heart and inoculated intra-ocularly into 3 rabbits. All 3 reacted with ocular signs, and from one of them (No. 1136) a strain was maintained in rabbits with ease, until discarded after the 8th generation (this strain is referred to below as the " daughter " strain, whereas the original " Seerangayee " strain used for inoculation of the gibbon is called the " parent " strain). The eye signs and the immunogenic characteristics of the daughter strain were those typical of the one tsutsugamushi and 5 rural typhus strains, all XK in type, previously studied by the authors (*vide* Parts I and II). Thus, from the 3rd generation onward 11 rabbits were inoculated, and all reacted with complete ocular signs—a result contrasting sharply with the non-survival of an X19 type virus beyond the 3rd generation by intra-ocular propagation, and with the earlier, shorter, and usually less intense ocular reactions that are caused by this virus (*vide* Part III). Two rabbits of the daughter strain (No. 1136 and 1161) were reinoculated intra-ocularly after 6 weeks' convalescence with virus of the parent strain ; both proved to be immune, while in each case a control rabbit developed a complete ocular reaction. Conversely, 2 convalescent rabbits of the parent strain were reinoculated intra-ocularly, along with 2 controls, with virus of the daughter strain ; both proved to be immune, while the controls reacted fully.

The serological reactions observed in the rabbits inoculated with the daughter strain were interesting. Of 3 rabbits inoculated with the blood of the gibbon, 2 gave a negative Weil-Felix reaction ; the 3rd (No. 1136) gave the following results :

Rabbit 1136 *: Agglutination Titres.*

Days after inoculation.	Living cultures. OXK.	OX19.
0	50	0
10	22	96
15	38	50
20	38	44
25	22	22

As noted above, it was by injection of infected aqueous humour from this rabbit that the daughter strain was continued. Two generations later another rabbit (No. 1161) gave a positive Weil-Felix reaction, OXK in type, thus :

Rabbit 1161 *: Agglutination Titres.*

Days after inoculation.	Living cultures.	
	OXK.	OX19.
0	25	0
10	50	0
15	125	0
19	110	0
22	50	0
28	50	0

These experiments with the daughter strain of virus thus showed that the antigenic component of the OX19 type, which was revealed by the serum reactions of the gibbon, was still demonstrable in the first generation of virus (Rabbit 1136), but no longer in the third generation (Rabbit 1161). In spite of the abnormal serum reactions of Rabbit No. 1136 this animal proved to be immune to reinfection with the parent strain of virus (type XK), and three other cross-immunity tests between parent and daughter strains confirmed their identity as regards immunogenic properties.

To afford more data the authors inoculated a second gibbon intradermally with the virus of the parent strain. This gibbon had, three months previously, reacted to intradermal inoculation of the virus of the tsutsugamushi disease with febrile reaction, leucopenia, dermal lesions, an agglutination of the OXK strain to a titre of 1/3850, and absence of any trace of agglutination of the OX19 strain. At the time of reinoculation the OXK agglutinins had fallen to zero. Peritoneal fluid from a reacting guinea-pig of the parent "Seerangayee" strain was inoculated at multiple sites into the gibbon, and into a macacus monkey as a control. In the gibbon leucopenia alone resulted ; no fever and no dermal lesions occurred ; there was no rise of agglutinins for OXK, and no trace of agglutination of OX19. It is known that failure to stimulate agglutinin production by reinoculation of a typhus virus is another indicator of complete cross-immunity obtaining between the virus strains used for the primary and the secondary inoculations (Felix, 1933). The control macacus monkey reacted with fever, dermal necrotic lesions, leucopenia and significant agglutination of the OXK type, but none of the OX19 type.

Two other macacus monkeys were inoculated intradermally with the virus of the parent strain ; both reacted typically, and their sera gave significant agglutination of the OXK type (as noted on p. 220), but no trace of agglutination of the OX19 type.

It appears justifiable to conclude, from the above data concerning the infection of the gibbon No. 2, that the passage from the guinea-pig through the rabbit to the gibbon of a virus of apparently pure XK type caused a temporary change in its agglutinogenic properties, but no change in its immunogenic properties.

SUMMARY.

1. The results of the Weil-Felix reactions of sera from rabbits and monkeys inoculated with human virus or virus of laboratory strains of rural typhus, urban typhus or the tsutsugamushi disease are reported and discussed; as also are corresponding results in rabbits inoculated with the virus of two strains of tropical typhus recovered from wild rats.

2. Instances are given of a change in type of antibody response; in particular, an experience is described in which the agglutinogenic properties of a virus of a tropical typhus strain underwent a temporary change, while the immunogenic properties remained unchanged.

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REFERENCES.

ANIGSTEIN, L.—(1933) "Studies on Tropical Typhus," *Stud. Inst. med. Res. F.M.S.*

FELIX, A.—(1929) *J. Hyg., Camb.*, **28**, 418.—(1933) *Trans. R. Soc. trop. Med. Hyg.*, **26**, 365.

Idem AND HEATLEY-SPENCER, J.—(1935) *J. R. Army med. Cps.*, **64**, 187.

FELIX, A., AND RHODES, M.—(1931) *J. Hyg., Camb.*, **31**, 225.

FLETCHER, W., AND FIELD, J. W.—(1927) *Bull. Inst. med. Res. F.M.S.*, No. 1 of 1927.

Idem AND LESSLAR, J. E.—(1926) *Ibid.*, No. 1 of 1926.

FLETCHER, W., LESSLAR, J. E., AND LEWTHWAITE, R.—(1929) *Trans. Roy. Soc. trop. Med. Hyg.*, **23**, 57.

LEWTHWAITE, R.—(1930) *Bull. Inst. med. Res. F.M.S.*, No. 3 of 1930.

Idem AND SAVOOR, S. R.—(1936) *Brit. J. exp. Path.*, **17**, 1.—(1936) *Ibid.*, **17**, 23.—(1936) *Ibid.*, **17**, 208.

PIPJER, A., AND DAU, H.—(1935) *J. Hyg., Camb.*, **35**, 116.

WEIL, E., AND FELIX, A.—(1921) *Z. ImmunForsch.*, **31**, 457.

WOLFF, J. W.—(1931) *J. Hyg., Camb.*, **31**, 352.

OBSERVATIONS ON THE POLYSACCHARIDE COMPLEX AND VARIANTS OF *VIBRIO CHOLERÆ*.

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In various communications (Yang and White, 1934; White, 1934*a* and *b*, 1935) I have reported the existence of three grades of polysaccharide structure in vibrios: the normal S form, presenting the full series of serologically active polysaccharide constituents; the R form, in which the element which characterizes the somatic serology of the intact S form is absent and in which a second polysaccharide hitherto masked is enabled to exert its serological activity; and thirdly, the ρ form, in which the dominant R polysaccharide being in turn suppressed, yet other serologically active substances containing carbohydrate are disclosed.

The series has now been investigated in greater detail, and it has become clear that, in each strain of *V. cholerœ* and seemingly in vibrios in general, at least four distinct groups of polysaccharide receptors or substances are concerned in the serology of the normal parent and variant forms. These specific carbohydrate substances may now be termed Cα, Cβ, Cγ and Cδ. To facilitate description, the main thesis that—

the S form possesses Cα, Cβ, Cγ and Cδ with Cα dominant,
the R form possesses Cβ, Cγ and Cδ with Cβ dominant,
the ρ form possesses Cγ and Cδ with Cδ dominant,

is stated at the outset. These named groups of receptors may be internally complex; the characteristic smooth receptors of the Inaba and Ogawa types of *V. cholerœ*—Cα (Inaba) and Cα (Ogawa)—are almost certainly so. A variant degraded below the level of the ρ form has not yet been discovered, but such forms possibly exist. The recognition of the four factors depends in the first place on the availability of suitable sera.

PREPARATION OF MONOVALENT POLYSACCHARIDE-PRECIPITATING SERA.

Anti-Cα sera.—Sera which (under the conditions of test) react exclusively with the homologous Cα are most certainly prepared by intravenous injection into rabbits of living vibrios or vibrios killed at 56° C. O-antisera prepared with vibrios heated at 100° C. are often satisfactory, but are very apt to show Cβ and Cγ antibodies. My method has been to give a rapid series of injections, 5–8 in 14–18 days, with bleeding some 5 days after the last injection.

Anti-Cβ sera are prepared by immunization of rabbits with rough vibrios. Here, again, strictly specific sera are most readily obtained by inoculation of living vibrios or vibrios killed at 56° C. over a short period. I have not

observed a production of Cγ precipitin in rabbits thus immunized with living R forms, and only traces at most after immunization with cultures killed at 56° C.; with much-heated cultures the antisera obtained are usually mixed in character.

Anti-Cγ and anti-Cδ sera.—The few sera obtained by inoculation of rabbits with living ρ vibrios have been specifically or predominantly anti-Cδ in character, and those prepared against vibrios killed at 56° C. have been as a rule similar. No direct and certain method has been found of obtaining a monovalent anti-Cγ serum. Such sera occasionally result from the injection of ρ vibrios long heated at 100° C., and one strictly specific anti-Cγ serum was obtained by inoculation of a similarly heated R form into a rabbit which, by some perversity and unlike another animal receiving identical inocula, failed to produce any Cβ-precipitin. As a rule, however, the sera obtained by immunization with much-heated R and ρ cultures are mixed in nature—anti-Cβ-Cγ or anti-Cγ-Cδ as the case may be. It may be said here that the use of a mixed anti-Cγ-Cδ serum introduces no serious complication in that Cγ and Cδ are readily separable, and in the precipitation test behave very differently. Further, I have found it perfectly feasible to produce monovalent Cγ and Cδ antisera by saturating the mixed antiserum with one or other of the polysaccharides.

SEPARATION OF Cα AND Cγ FROM Cβ AND Cδ.

Mass cultures of agar-grown vibrios are finely ground in alcohol, boiled therein and filtered off. The alcohol-washed vibrios are suspended in saline (the growth from 5 sq. feet of agar surface, in 200–250 c.c.) and boiled for 15–20 minutes. The suspension is cooled to 60–65° C., and 1 p.c. of papain, in fine suspension, is added, rendering the reaction definitely acid, pH 6–5. Digestion is allowed to proceed at this temperature for 6–7 hours and at room temperature overnight. Next day the mixture is centrifuged at high speed.

Supernatant fluid.—The supernatant contains the great bulk of Cγ and, in the case of S cultures, most of Cα also. It is heated to 100° C. for 20 minutes, and the precipitate, which does not, as a rule, contain much active material, is removed. In dealing with S cultures, where any severe exposure to alkali is inadmissible, owing to the susceptibility of Cα, the supernatant is heated for half an hour at 100° C. with $N/20$–$N/10$ acetic acid and the precipitate which forms is removed by centrifuging.

The active material is precipitated from slightly acid or neutral solution with 2½–3 volumes of alcohol. (Absolute ethyl alcohol containing 5 p.c. of methyl alcohol has been employed as the cheapest spirit available.)

When R and ρ cultures are in hand, the primary extracts do not contain the alkali-labile factor, Cα, and there is no ban on the use of alkali in their case. They may, therefore, be heated with $N/10$ NaOH solution to assist separation of Cγ from protein. Any precipitates formed on alkalization or on later acidification with acetic acid are removed, and the active substance is precipitated from alkaline solution with 2½ volumes of alcohol. The precipitate is redissolved, acidified with acetic acid and reprecipitated with alcohol. The product thus obtained is a crude protein-containing fraction, designated Fraction I.

Deposit.—The deposit from the digestion mixture contains Cδ and in the case of S and R organisms the whole of Cβ also; traces of Cα and Cγ may also be left behind. The deposit is emulsified in about 100–120 c.c. saline and heated for 20–30 minutes at 100° C. with $N/4$ NaOH; thus most of the Cα present is inactivated. The mixture is centrifuged, the precipitate removed, and the supernatant mixed with $3\frac{1}{2}$ volumes of alcohol. The active substances at issue are then precipitated. By adding the alcohol in stages it is sometimes possible to effect some fractionation, removing in the first precipitates most of the Cγ contaminant. The precipitate is redissolved; the solution is acidified with acetic acid and clarified, and the active material is reprecipitated with alcohol, giving crude fraction II.

The crude fractions I and II are now extracted with saturated aqueous solution of picric acid—about 100 c.c. in each case. The resultant extracts, cleared by centrifugation and filtration, are mixed with $3\frac{1}{2}$–4 volumes of alcohol to precipitate the active substances. Addition of a little salt assists flocculation. The precipitates are collected, redissolved and reprecipitated with alcohol. The process is continued till neither precipitate nor washings show a yellow tinge.

The fractions are, in each case, made up in 40–50 c.c. of saline rendered alkaline—slightly so in the case of Cα-containing fractions, strongly so in other cases—and, after any precipitate has been removed, reprecipitated with alcohol. After further precipitation from acid and then from neutral solution the fractions are washed with absolute alcohol and ether and dried. The precipitate formed on alkalization of fractions I, R and ρ has as a rule shown relatively little serological activity, and has been discarded, but that obtained from fraction I S has often shown a considerable concentration of Cα activity, and in such cases it has been separately worked up as fraction I, Cα.

In this way are obtained from each culture two main fractions:

	Fraction I.	Fraction II.
From the S form . . .	Cα + Cγ .	Cβ + Cδ
From the R form . . .	Cγ .	Cβ + Cδ
From the ρ form . . .	Cγ .	Cδ

Fraction II is sometimes found to contain traces of fraction I, but as a rule contaminating activity is "lost" during the precipitation process.

While the method given is fairly efficient as regards extraction of Cβ, Cγ and Cδ, much of Cα is lost during deproteinization of fraction I. It is hoped to correct this defect by achieving fuller liberation of Cα at the initial stage.

The differential action of papain in liberating the various C substances suggests that the association of these with protein is very different. The fractions are biuret-negative, give an intensely positive Molisch reaction, and yield reducing substances on hydrolysis with mineral acid—Cβ readily, Cα less readily, Cγ only after vigorous treatment.

I have to thank Miss Llewellyn Smith of this Institute for determining the nitrogen contents of a series of fractions derived from the S, R and ρ races of a *V. choleræ* strain. Fractions I showed respectively 4·5, 3·3 and 4·6 p.c. of N; fractions II, 3·3, 4·6 and 3·1 p.c. Whether these values are in any way definitive remains to be seen, when further purification is attempted. It may

well be that the fractions so far obtained are heavily contaminated with inactive material.

In extending the term "polysaccharide" to all these four substances, without exact knowledge of their intimate structure I am fully conscious that the term may be to some extent or in some cases a misnomer. It is hoped to gather more accurate information as to their nature.

DIFFERENTIAL PRECIPITATION REACTIONS OF THE FRACTIONS OF *V. CHOLERÆ*.

In this communication the logical order of argument has to some extent been reversed and it remains to justify the statements made. This may, I think, be adequately done by a qualitative summary (Table I) of results obtained in the study of two strains of *V. cholerœ*, Shillong 1077 (type Ogawa) and Inaba (type Inaba) in their S, R and ρ forms, supplemented by observations on the S and R races of the Ogawa type strain Kasauli $92/_1$ and other typical O group I strains. Apart from cases in which fraction II has been appreciably contaminated with the active substances of fraction I—a clearly recognizable occurrence—the results throughout have shown perfect regularity and definition. Activity associated with fraction II has never been encountered in fraction I. The distinct serological properties of fractions I and II are clear from a study of the table. The activities of Cγ are readily recognizable, wherever they occur, by the fact that in "ring tests" the precipitate, however feeble, quickly falls downwards into the serum; all other "rings", unless extremely intense, maintain their position for hours, or tend to extend upwards into the polysaccharide solution.

TABLE I.

Form of *V. cholerœ* from which test material was derived.	Material tested.	Monovalent serum. Anti-S (Cα) (type homologous).	Anti-R (Cβ).	Anti-ρ (Cγ).	Anti-ρ (Cδ).
S	Fraction I	++	−	++*	−
	" treated NaOH	−	−	++*	−
	" separated Cα	++	−	(tr.* or +*)	−
R	"	−	−	++*	−
ρ	"	−	−	++*	−
S	Fraction II	−	++	− or (tr.*)	+
R	"	−	++	− or (tr.*)	+
ρ	"	−	−	− or (tr.*)	++

++ or ++* = positive "ring" precipitation test in 1 : 20,000 (or lesser) concentration.
+ or +* = " " " " in 1 : 5000, but not in lesser concentration.
tr. = " " " " in 1 : 500–1 : 1000, but not in lesser concentrations.
− = "ring" precipitation test negative in 1 : 500 and lesser concentrations.
Brackets indicate that the reactions are regarded as due to impurities and were variable with different samples.
* indicates the peculiar reaction of Cγ.

From the S organism and from this only is obtained material reacting with true smooth antiserum, and the material concerned is, unlike any of the other factors concerned, rapidly inactivated by hot $N/10$ or $N/5$ NaOH. There is some reason to suppose that this inactivation proceeds by two or more qualitative steps. In the case of S anti-cholera sera which do not sharply differentiate

between the Ogawa and Inaba types there is little selection in the precipitating action on fraction I obtained from either type. Those sera, however which effect a fairly sharp differentiation of the types react relatively more strongly with the homologous than with the heterologous S fraction I. S fraction I exhausts the type homologous O agglutinins almost completely.

Fraction I never reacts with strictly R antisera, but often does so with sera prepared against much-heated R forms, and then gives the same characteristic precipitate which it regularly forms with anti-γ sera, whether it be derived from S, R or ρ vibrios. There can be no doubt of the presence of two distinct receptor groups (Cα and Cγ) in fraction I. To this I drew attention recently (White, 1935)—then treating, I now think wrongly, Cγ as the characteristic R substance. The marked concentration of Cα activity in precipitates formed on alkalization of fraction I S solutions shows quite clearly that Cα exists independently of Cγ.

Fraction II does not react with smooth antiserum, though this does not preclude the possibility that it contains, when derived from S forms, traces of inactivated Cα. As a rule small quantities of material, reacting with strict anti-Cγ sera and discernible at the outset of purification, are " lost " during the precipitation processes. With strict anti-R (Cβ) sera fraction II derived from either S or R forms reacts strongly in precipitation tests ; that from ρ forms not at all. Some anti-R sera, prepared against boiled cultures, give a slight reaction with ρ fraction II ; such sera, in my experience, give a patently double " ring " in tests with R fraction II. It was this double " ring " which first led me to suspect that the slight reaction of the ρ fraction II with some anti-R sera was not due to traces of Cβ, but to a fourth factor included with Cβ in fraction II R. A study of various anti-ρ sera fully confirmed this opinion.

Though a very large number of antisera for S, R and ρ races of *V. cholerœ*, prepared against living and much-heated vibrios, have been tested with the numerous polysaccharide fractions prepared, I have not yet found evidence in the latter of any type of serological activity not included in the schema presented. It is hardly to be thought, however, that the fractions obtained are single substances or simple mixtures of the reacting substances described. It is probable that fraction I in particular contains a considerable amount of matter inactive in serological tests.

That the antisera employed in these tests possess various precipitating properties—notably against proteins—other than those of which use has here been made is very evident ; whether any of these other antibodies and the antigens on which they depend are involved in the S–R–ρ transition has yet to be determined.

It should be said that all the sera employed were tested for agar-precipitins and found free.

A study of vibrios representative of the various agglutination types has been begun. It will suffice to say here that the true El Tor vibrio presents a polysaccharide complex serologically identical with that of *V. cholerœ* (of the same absorption type) ; that the Cγ and Cδ factors seem to be common, so far as can be judged by simple precipitation tests, to all the types of vibrio so far examined ; that different groups of vibrios show sharp differences in the

behaviour of their Cβ substances; and that the Cα substances determine the serological specificity of the various smooth types.

SUMMARY.

1. The smooth cholera vibrio possesses four serologically active polysaccharide or non-protein carbohydrate-containing constituents—here termed Cα, Cβ, Cγ and Cδ. Of these, Cα, the determinant of smooth-type specificity, is lost in roughening, exposing Cβ, the characteristic rough polysaccharide. On degradation to the ρ form Cβ disappears, but Cγ and Cδ—substances common in whole or in part to many vibrios—remain.

2. On digestion of cholera vibrios with papain in a slightly acid medium Cα and Cγ are brought into solution; Cβ and Cδ are not, but may be liberated from the residue with alkali.

REFERENCES.

WHITE, P. BRUCE.—(1934*a*) *Bull. Off. int. Hyg. publ.*, **26**, No. 7, Suppl., p. 73.—(1934*b*) *J. Path. Bact.*, **39**, 530.—(1935) *Ibid.*, **41**, 567.
YANG, Y. N., AND WHITE, P. BRUCE.—(1934) *J. Path. Bact.*, **38**, 185.

THYMUS AND ADRENALS IN THE RESPONSE OF THE ORGANISM TO INJURIES AND INTOXICATIONS.

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IN conditions of intoxication by drugs, some organs react more readily than others. The thymus, the adrenal and the liver seem to be particularly sensitive. Diseases or experimentally-produced lesions of the adrenal or of the liver decrease the resistance of the organism to numerous toxic substances. These findings made it very likely that the thymus, the adrenal and the liver play an important role in the defence reaction of the organism against drugs and traumatic injuries. In the present communication experiments are described which were designed to clarify some of the inter-relations which exist between these three organs after traumatic injuries and during various intoxications.*

* A short summary was presented at the Washington Meeting of the Federation of the American Societies for Experimental Biology, March, 1936.

EFFECT OF FASTING, VARIOUS OPERATIVE INJURIES AND DRUGS.

The fact that various drugs, infectious and other diseases may cause a sudden so-called " accidental " involution of the thymus has long been known, but it has also been established that quantitatively and qualitatively inadequate nutrition will produce similar results. Both malnutrition and intoxication will also cause adrenal enlargement and decrease in liver size. It seemed important, therefore, to establish what part malnutrition plays in the production of the changes occurring in these three organs during various defence reactions of the organism, for it is obvous that any severe injury will decrease the appetite of the animal and thus cause malnutrition.

The normal variations in size of these organs are considerable, and are greatly influenced by age, sex, diet and various environmental conditions. I used, therefore, inbred female rats of the same colony, 2–5 months old, fed on a uniform " Purina " diet ; but even within this age-range one can only compare groups born in the same month, for drugs will lead to much more rapid thymus involution in 5-months-old animals than in 2-months-old ones. Owing to the great individual variations, particularly in thymus size, we had to draw our conclusions from averages of large experimental series, so that over 1500 rats were necessary to complete this work. It is impossible to report on each individual experiment without prolonging this paper unduly, and only representative, sample experiments, necessary to substantiate our conclusions, will be discussed.

In order to establish the effect of fasting on the weight of thymus, adrenal and liver in the rat, four groups of normal females were fasted for 1, 2, 3 and 4 days respectively. The weights of the organs in question at the end of these periods are reported in Table I, together with normal controls. These data show that no considerable decrease in thymus size occurs until the end of the second day, and that on the other hand animals fasted for 4 days or longer invariably show pronounced thymus involution. I concluded, therefore, that any experimental condition which will produce pronounced thymus involution (that is, an average thymus weight below 100 mg.) within 48 hours acts in itself, and not simply by decreasing the appetite. It is evident, furthermore, that since 4 days' fasting will invariably produce severe thymus atrophy, any experimental condition which will maintain the thymus weight at its normal level in an animal fasted for this length of time must have an inhibiting effect on thymus involution.

After a few preliminary experiments with various drugs, we found that atropine, morphine and formaldehyde are particularly active in producing rapid thymus involution. As may be seen from Table II, atropine in doses of 2 c.c. of a 1 p.c. solution twice daily subcutaneously, or morphine in doses of 2 c.c. of a 1 p.c. solution twice daily subcutaneously, or formaldehyde in doses of 0·5 c.c. of a 4 p.c. solution twice daily subcutaneously, invariably produce severe thymus involution within 48 hours after the first injection. Since 48 hours' fasting has no marked effect on thymus size, we fasted our experimental animals during the injection period, so as to avoid variations due to differences in the amount of food consumed. From the same table it may also be seen that the adrenals enlarge quite rapidly, and the increase in

TABLE I.

Normals.	Thymus weight (mg.).						Adrenal weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
2 months old. Fed.	187	318	340	325	295	321	31	34	42	36	30	26	112	138	163	162	136	125
3 „ „ „	190	315	241	298	300	340	56	44	68	50	46	36	159	164	183	162	156	138
5 „ „ „	171	150	152	304	205	160	52	48	38	56	32	56	182	161	156	190	168	174
2½ „ „ Fasted 24 hrs.	193	174	254	213	293	235	34	26	34	39	37	39	109	106	113	170	120	113
3 „ „ „ 24 „	170	195	161	248	183	212	39	52	42	53	41	46	157	166	170	140	132	163
2 „ „ „ 48 „	151	203	228	161	190	220	44	45	70	51	51	46	134	130	180	140	127	150
3 „ „ „ 48 „	150	135	141	104	140	203	44	40	41	34	39	60	150	208	154	138	155	178
5 „ „ „ 48 „	146	121	71	61	120	105	44	50	49	51	48	40	176	157	190	155	159	160
3 „ „ „ 72 „	113	20	92	95	58	132	50	48	54	38	50	40	139	120	160	130	120	145
3 „ „ „ 96 „	67	70	115	77	60	90	54	60	58	62	49	50	145	164	153	148	120	170

TABLE II.

Normals.	Thymus weight (mg.).						Adrenal weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Atropine sulphate: 2 c.c. of 1 p.c. solution twice daily for 48 hours. 3 months old	37	58	35	38	74	90	54	70	66	71	75	48	136	147	122	145	160	155
Morphine sulphate: 2 c.c. of 1 p.c. solution twice daily for 48 hours. 3 months old	88	85	60	70	105	62	40	46	50	40	60	60	124	171	105	115	135	158
Formaldehyde: 0·5 c.c. of 4 p.c. solution twice daily for 48 hours. 3 months old	77	85	78	20	65	58	60	58	58	70	58	57	168	210	144	197	153	156
Adrenaline (1/1000): 0·2 c.c. twice daily for 48 hours. 3 months old	123	73	23	112	107	67	78	40	58	42	40	45	173	120	112	150	141	113
Adrenaline (1/1000): 0·1 c.c. twice daily for 48 hours. 5 months old	72	60	95	85	80	86	60	58	64	56	60	50	170	193	183	161	171	172

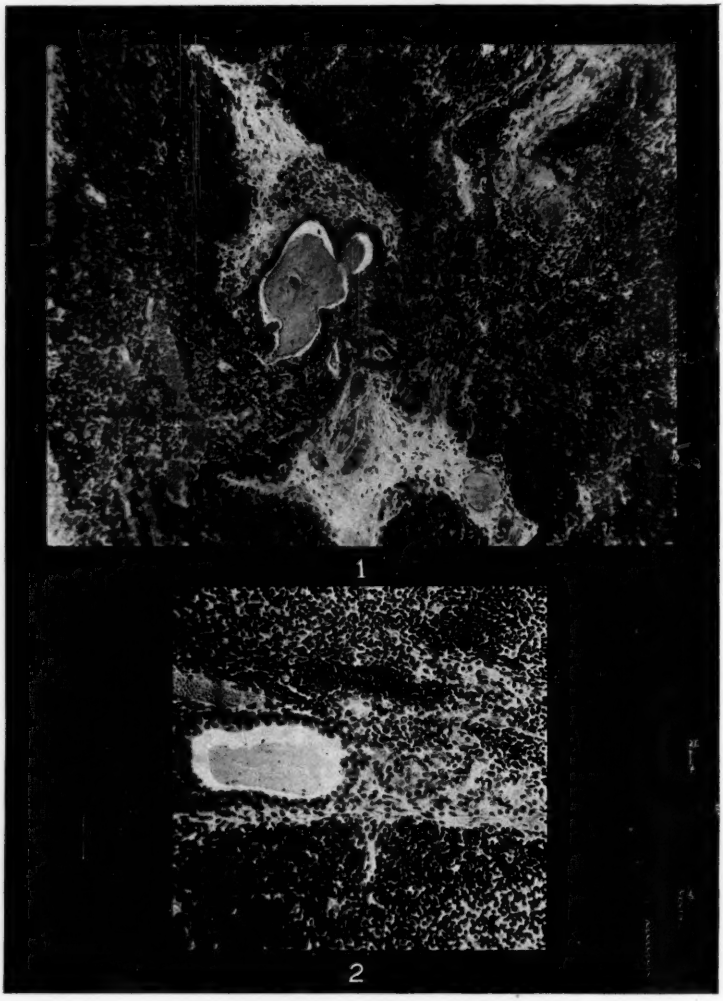

FIG. 1.—Thymus of a rat 48 hours after initiation of morphine treatment. Intrathymic epithelial structure: on the left vesicles filled with colloid resembling thyroid tissue; on the right massive strands of polygonal epithelial cells of "parathyroid-like" appearance.

FIG. 2.—Thymus of a rat treated in a manner similar to that in Fig. 1, showing again typical vesicle formation on the left and massive epithelial strands on the right.

their size is very closely proportionate to the decrease in thymus size. The lipoid content of the adrenals is decreased during this acute hypertrophy, so that their colour is brown and not yellow as it is normally. This loss of lipoids is a symptom of increased activity, according to various authors. It is of interest to note that definite changes in the thymus are already detectable as early as 6 hours, and sometimes even sooner, after the administration of these drugs. The first reaction is a considerable œdema of the organ, which causes an apparent increase in its size during the first hours following the injection. At this time some fluid is usually found in the pleural cavity, and a more generalized tendency to œdema formation manifests itself by the occasional appearance of retroperitoneal œdema.

Histological examination of the thymus after injection of these drugs shows: 24 hours after involution begins the thymocytes degenerate and the chromatin of their nuclei disintegrates into free basophilic granules, which are the most characteristic feature of this first stage of involution. The interlobular connective tissue is œdematous, and degenerative changes appear also in the blood-vessels of the organ.* The lymph-glands of the thymus are usually enlarged, and their sinuses are filled with large wandering cells containing nuclear debris of thymocytes and red blood-cells. Hæmorrhages into the thymus tissue are also frequently observed. After 48 hours the œdema usually subsides and the nuclear debris begins to disappear from the gland. At this stage and later tubular epithelial structures appear quite frequently in the thymus. They may contain eosinophilic colloid and resemble thyroid tissue, or they may be solid. In the latter case their resemblance to parathyroid tissue is so striking that one wonders whether the so-called accessory parathyroids found in the thymus might not be identical with these bodies. The photograph of an intrathymic accessory parathyroid in Spreter's (1935) publication, for instance, is certainly very similar to the epithelial structures we see in involuting thymuses (see Figs. 1 and 2). In a normal, well-developed thymus we have not seen such structures. Nothing can as yet be said about their function, but their glandular structure in the absence of an excretory duct makes them very similar to endocrine tissue. In cases of very advanced involution, almost the entire gland consists of extremely atrophic cells, with very small and intensely basophilic nuclei. This "basophilic" atrophy may occasionally be seen in small areas of normal thymuses, but in these it is always limited to little patches. This patchy basophilic atrophy is not uncommon in other glands such as the thyroid and the parathyroid.

The marked enlargement of the adrenals, and the fact that adrenaline secretion has frequently been found to be increased after administration of toxic substances, made us wonder whether the thymus involution is not simply due to hyperadrenalinæmia. In order to test this hypothesis, we had to establish first whether adrenaline would have the same effect as the other drugs used. The data in Table II show that adrenaline in doses of 0·2 c.c. of a 1/1000 solution twice daily subcutaneously will cause just as severe and rapid thymus involution as the other drugs previously used. But this finding

* We believe that this change is identical with the "Crise caryoclasique" of Dustin (1921), but we do not feel that it represents a specific reaction to certain drugs with "actions caryoclasiques", since we observed the same changes after surgical injuries even though no drugs were injected.

in itself is of course no proof that adrenaline plays any role in the causation of thymus involution by other drugs.

Since we have frequently seen in the course of other experimental series that the thymus of animals autopsied soon after a major operation is usually very small, we wished to establish whether this post-operative involution is simply due to decrease in appetite, or to some more specific influence of the surgical shock on the thymus. Table III shows the effect of various operative injuries on the thymus of animals fasted during the 48 hours following operation. From these data we see that extensive skin-lesions (the skin has been detached from the subcutis over large areas), bone fractures (tibia and femur were fractured under anæsthesia on both sides), peritoneal injuries (all the intestines were placed outside the peritoneal cavity for one minute), and even the mere excitement of an animal the free motion of which is interfered with (by tying the legs together or wrapping the animal tightly in a towel), will cause more or less pronounced thymus involution. At the same time the adrenals are enlarged and usually free from lipoids.

A group of 12 rats kept at a low temperature varying from +1 to 7° C. showed much more marked thymus involution and adrenal enlargement after 48 hours than their fasting controls. The effect of high temperature (35–40° C.) on the thymus was less marked, however. As an incidental observation, it may be of interest to mention that with one exception all the animals kept at a low temperature of +1 to 3° C. developed gastric ulcers. This was also the case in another series of 8 rats kept at a temperature of −2° C. during 24 hours. It is tempting to suspect some correlation between the so-called "hyper-adrenalinæmia of excitement", causing the well-known hyperglycæmia of excitement, and the adrenal enlargement and thymus involution of our experiments.

EFFECT OF ADRENALECTOMY ON THYMUS INVOLUTION.

It seems evident from our tables that the drugs and operative procedures which produce thymus involution almost invariably lead to hypertrophy of the adrenals, and the size of these two glands is approximately in inverse proportion. It appeared to be of interest, therefore, to investigate the possible *role* that the adrenals may play in the process of thymus involution. For this purpose a study of the effect of some injuries and drugs on the thymus of adrenalectomized rats was made. Naturally only such drugs and injuries were used which were found to produce thymus involution in experiments on normal animals. Table IV summarizes the results of this series.

It appears that, in the absence of the adrenals, none of the drugs and injuries which would lead to thymus involution in normal animals will have any effect on the thymus. A secretion of the adrenal gland must therefore be considered essential for the ability of the thymus to undergo sudden involution.

Some indications of a thymo-adrenal inter-relationship have already been pointed out in the literature. Thus it has repeatedly been stated that the thymus enlarges during adrenal insufficiency. Star (1895) was probably the first to call attention to the fact that thymus hypertrophy occurs in Addison's

Table III.

Injuries.	Thymus weight (mg.).						Adrenal weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Skin lesion: 48 hours. 3 months old. Fasted	83	151	80	128	78	102	48	45	41	50	49	38	165	136	155	190	155	138
Bone lesion: 48 hours. 3 months old. Fasted	103	203	148	169	92	153	44	46	54	48	57	48	132	153	144	128	147	151
Legs tied: 48 hrs. 3 months old. Fasted	30	90	96	71	75	131	79	74	40	78	44	94	192	197	140	170	171	209
Legs tied: 48 hrs. 5 months old. Fasted	84	95	82	105	65	118	44	60	50	58	41	60	159	196	189	160	171	161
Wrapped in towel: 48 hours. 5 months old. Fasted	120	25	80	114	67	72	60	61	64	54	56	51	197	190	160	170	159	159
Exposed to cold, 1–3° C.: 48 hrs. 5 months old. Fasted	56	82	35	40	48	41	56	70	52	48	50	50	152	151	179	156	152	179
Exposed to heat, 35–40° C. 48 hrs. 5 months old	106	99	58	104	131	81	52	50	42	48	40	39	203	189	173	169	165	151
Peritoneal lesion: 48 hrs. 3 months old	145	79	85	115	80	95	50	72	52	74	82	69	185	153	167	135	193	152
Medulla extirpated: fasted 96 hrs. 5 months old	180	72	81	101	185	156	—	—	—	—	—	—	154	153	143	147	153	167
Exposed to cold, 5–7° C. 48 hrs. 3 months old. Fasted	115	130	75	125	89	77	68	69	59	71	67	54	149	181	142	149	137	152

Table IV.

Adrenalectomized: On water.	Thymus weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Legs tied: 3 months old. Died within 36 hrs.	267	167	287	231	190	163	153	134	129	139	172	143
Formaldehyde: 0·5 c.c. of 4 p.c. solution twice daily. 3 months old. All died within 48 hrs.	254	156	228	210	183	171	168	132	150	145	163	145
Adrenaline (1/1000): 0·2 c.c. twice daily. 3 months old. All except Nos. 1 and 4 died within 48 hrs.	199	178	196	198	270	183	133	140	143	130	144	157
Controls: adrenalectomized. 3 months old	200	226	244	218	248	198	150	134	130	144	132	145
2 months old. Fasted 48 hrs.	208	275	248	188	231	175	126	159	134	132	137	132
2½ months old. Fasted 96 hrs.	241	196	225	240	225	230	110	95	120	110	115	174

disease, and this was later confirmed by other authors. Boinet (1899) reported that thymus enlargement may be elicited by adrenalectomy, and the same has been found since by many others. We find no data in the literature concerning the effect of adrenalectomy on the ability of the thymus to undergo accidental involution, except a statement made by Jaffe (1924) that adrenalectomized rats dying within 5–6 days after operation have thymuses 39 p.c. below normal in size, and this is attributed to the fact that "suprarenalectomized animals succumbing gradually in the course of some days do not take food and consequently thymus involution due to inanition and intoxication manifests itself". Another relevant statement is made by Marine *et al.* (1924), who state that thyroidectomy not only prevents the hyperplasia of the thymus in the adrenalectomized rabbit, but actually causes involution in spite of the absence of adrenal tissue. They conclude: "The thyroid hormone is therefore necessary for thymus hyperplasia which usually follows sufficient but sublethal injury of the suprarenal function." A careful study of the papers referred to above and of our own experimental findings makes it very doubtful whether thymus hyperplasia in the strict sense of the word ever occurs after adrenalectomy. Let us take, for instance, the very exact study of Jaffe, which we are in a better position to discuss than that of others, since it was done on the rat, and gives an accurate description of the experimental conditions. From his data it is evident that the thymus of suprarenalectomized rats killed at the age of 208 to 212 days is much larger than that of normal controls of the same age, but its weight is not higher than that of normal rats at the age of 30 to 90 days. Our experiments seem to indicate that the action which the adrenal exerts on the thymus is fully explained by the assumption that it makes the sudden so-called accidental involution of the latter possible. In the absence of the adrenal gland, all those stimuli which cause thymus involution in normal animals are inert. It seems quite likely, therefore, that the effect of adrenalectomy in the hands of previous investigators was only to restore the thymus to a size which equals (or almost equals) the size which the organ usually has at the time of its full development. In normal animals, age, and all the incidental damaging influences which so easily affect the size of the thymus, cause it to involute more and more with time; but all these stimuli act through the adrenals, and are inactive in their absence, so that the thymus will be much larger after adrenalectomy than that of normal animals of the same age.

The question arose which of the adrenal hormones is responsible for the effect which this gland has on the thymus. Since adrenaline secretion is known to be induced by various stimuli which lead to excitement, and a great number of drugs, we first thought that this might be the hormone in question. This suspicion was further corroborated by the finding reported above—that adrenaline itself is extremely active in causing thymus involution. In order to test this theory, one would have to show that adrenaline will cause thymus involution in the adrenalectomized animal. From Tables IV, V and VI we see, however, that this is not the case. Adrenalectomized animals are extremely sensitive to adrenaline, and most of them die during such an experiment, but even those which live long enough to be significant show no thymus involution. From this we would conclude that it is not adrenaline which causes thymus involution in the normal animal. As this is one of the most important points

TABLE V.

Adrenalectomized: Saline given 24 hours before and during experiment.	Thymus weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
2½ months old. Fasted 96 hrs.	282	241	162	185	262	140	95	131	100	100	95	100
Legs tied: 3 months old. All lived 46 hrs.	135	212	115	142	187	180	159	139	189	145	145	130
Formaldehyde: 0·5 c.c. of 4 p.c. solution. 48 hrs. 3 months old	175	225	175	168	230	139	140	123	127	147	170	133
Controls: adrenalectomized. 3 months old; given food and saline 48 hrs.	277	190	241	130	277	222	141	134	138	137	120	144
Adrenaline (1/1000): 0·2 c.c. twice daily 48 hrs. 3 months old	60	242	293	231	244	260	100	180	145	220	173	217
Formaldehyde: 0·5 c.c. of 4 p.c. solution 96 hrs. 3 months old	290	325	176	115	194	225	142	144	120	123	115	119
Adrenaline (1/1000): 0·2 c.c. twice daily 96 hrs. 3 months old	220	240	205	237	167	310	138	135	118	120	140	143

TABLE VI.

Adrenalectomy + adrenaline + cortin.	Thymus weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Adrenaline (1/1000) 0·2 c.c. twice daily + cortin 1 c.c. per day, 48 hrs. 2½ months old. One died after 3 injections	183	173	213	140	188	199	134	144	147	128	148	130
Adrenaline (1/1000): 0·2 c.c. twice daily + cortin 0·5 c.c. per day, 48 hrs. 2 months old. One died. Saline given	198	181	224	210	200	275	139	142	122	148	141	131
Cortin: 4 c.c. per day. 48 hrs. 2 months old	146	225	170	—	—	—	128	111	137	—	—	—
Adrenaline (1/1000): 0·2 c.c. twice daily + cortin 4 c.c. per day, 48 hrs. 2 months old	205	242	145	148	—	—	116	138	127	135	—	—

for the interpretation of our experiments we wished to obtain more evidence for it.

Treatment with sodium chloride is known to improve the general condition of adrenalectomized rats considerably, so that their body-weight and even their sexual cycles will be maintained. From Table V we see, however, that even though salt-treated adrenalectomized animals maintain their normal resistance to toxic doses of various drugs, their thymuses will be unable to involute after fasting, toxic doses of drugs, and various surgical injuries. The salt was administered in the form of an 0·9 p.c. NaCl solution, which was given instead of drinking-water. With the help of salt treatment, it was possible to maintain adrenalectomized rats treated with 0·2 c.c. of a 1/1000 solution of adrenaline twice daily subcutaneously over long periods, and we found that even 4 days' treatment with this hormone caused no thymus involution. Since even 48 hours' treatment would have decreased the weight of the thymus considerably, in the presence of the adrenals, we cannot consider adrenaline as the adrenal hormone acting on the thymus.

The experiments with salt-treated rats, on the other hand, show that the resistance to drugs is almost completely restored to normal. Numerous experiments with various drugs such as morphine, atropine and adrenaline—not all of which are included in our table—showed that even doses high enough to kill a considerable percentage of the normal animals will not kill a much higher percentage of adrenalectomized, salt-treated rats. This seems to be of interest particularly because it is known that adrenalectomy has a specific effect on the resistance of animals to toxic substances, and the restoration of their resistance has repeatedly been used as a means of assaying cortical hormone preparations (Perla and Marmorston-Gottesman, 1931, and Leloir and Novelli, 1933). Gaunt and co-workers (1934) think that the development of accessory adrenal tissue plays a great part in helping animals to survive for a long time when treated with salt. Since our experiments were performed on the second day after adrenalectomy, development of accessory tissue could not yet have taken place. It may be of some help in explaining the mechanism of salt action to note that the great efficiency of sodium chloride on the restoration of drug resistance was observed only in fed animals; fasted adrenalectomized animals were just as susceptible to drugs whether they received salt treatment or not. We should like to mention in this connection that similar findings have been made in cases of Addison's disease, in which salt treatment proved to be efficient only if the patient took sufficient nourishment (Browne, personal communication).

After adrenaline and salt treatment proved ineffective in restoring the ability of the thymus to involute, it was necessary to establish whether cortin would have this effect. *A priori*, it would have been possible that either cortin or a combination of cortin and adrenaline would be effective. In order to test this possibility, we gave 0·2 c.c. of adrenaline (1/1000) subcutaneously twice daily for 4 days to a group of adrenalectomized rats kept on salt treatment and injected subcutaneously with 5–10 Swingle and Pfiffner dog-units of cortin daily,* the total amount being divided into 4 doses. Such protracted

* We are greatly indebted to the Connaught Laboratories in Toronto for the cortical extract supplied for these experiments through the courtesy of Prof. C. H. Best.

administration increases the activity of a given dose of cortin greatly, as shown by Kutz (personal communication), in the rat. The amount of adrenaline given in this experiment would have produced thymus involution within 48 hours in normal animals, as our experiments show; in these adrenalectomized animals, however, no thymus involution was seen after 4 days of treatment, even though cortin and salt were given. These doses of cortin were large anough to restore the adrenalectomized animal's resistance to adrenaline, but apparently had no effect on the thymus. In order to see whether still larger doses of cortin would have any effect, 8 c.c. of the cortical extract containing 10 dog-units per c.c. were injected in divided doses within 48 hours to 4 adrenalectomized, 2 months old, female rats receiving 0·2 mg.

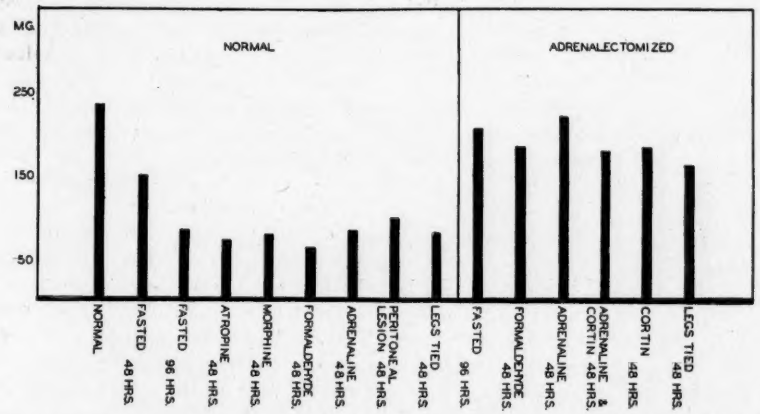

FIG. 3.—The black areas indicate the weight of the thymus under the various experimental conditions, and have been obtained from averages of large groups of rats. It would appear that the thymus of untreated animals is even larger than that of adrenalectomized, treated ones; but owing to the great individual variations it is questionable whether this is significant. It is obvious, at any rate, that those stimuli which produce thymus involution in normals are not active in the absence of the adrenals.

of adrenaline twice daily at the same time, and to 3 others also adrenalectomized but not treated with adrenaline. Even these enormous doses of cortin were not able to cause a thymus involution of the same order as that obtained in the non-adrenalectomized rat. The average weight of the thymus, however, was slightly below that of the untreated controls; but considering the unusually large quantity of extract administered in this series, it is hardly possible to decide whether this slight decrease in weight is due to cortin itself or to traces of a contaminating substance in the extract. The results of these experiments are summarized in Table VI.

In order to see whether the medulla of the adrenal is essential for the action of this gland on the thymus, we removed the adrenal medulla in 6 rats. After making a small incision through the cortex, the borders of the wound were everted by pressure on the opposite pole of the gland; this

exposed the medulla in a very satisfactory way. With the help of a very thin capillary glass tube conneeted with a suction pump, we proceeded to remove the soft and brittle medullary tissue by suction. This allowed of removal of the medulla without seriously injuring the cortex. In fact histological examination of such glands showed that the cortical tissue was well vascularized and alive, while the medullary cavity was filled with necrotic tissue only. If such animals are fasted for 4 days and then autopsied on the 5th day no marked thymus involution occurs, which seems to show that the medulla is essential for the action of the adrenal on the thymus (see Table III). These findings would seem to indicate that the adrenals play a part in the accidental involution of the thymus which is not wholly explicable in terms of secretion of either or both of the known adrenal hormones, adrenaline and cortin. However, one has to consider the possibility that the amount of adrenaline and cortin necessary to produce thymus involution is even larger than the enormous doses which we used. In this case one would have to assume that more than 80 dog-units of cortin are produced by the adrenal of the rat within 48 hours, under the influence of such conditions as have been found capable of producing thymus involution during this time. The results of the part of our experiments thus far discussed are summarized in Fig. 3.

EFFECT OF HYPOPHYSECTOMY ON THYMUS INVOLUTION.

Since the adrenal is known to be largely dependent upon pituitary function, we were interested to see whether hypophysectomy would have any effect on the ability of the thymus to involute. The publications concerning the effect of hypophysectomy on the thymus are rather contradictory. There are no data in the literature concerning the ability of the thymus to undergo involution under the influence of external stimuli.

In a series of experiments on rats hypophysectomized 8 days before the experiment was started, we studied the effects of such damaging influences as would produce thymus involution in normal animals. Table VII summarizes our findings. We see that hypophysectomy produces only very slight thymus involution, if any, in the rat, and that the thymus of hypophysectomized animals does not undergo as marked accidental involution as that of normals. It seems, however, that a certain degree of involution may occur in spite of the absence of the hypophysis. It is possible that the sudden breakdown of the adrenals after hypophysectomy would liberate such hormones from the gland as are necessary for the production of thymus involution. This might explain the rather contradictory findings reported in the literature. Another possibility to be considered is that a short period of increased activity may follow hypophysectomy in glands standing under pituitary control. At any rate our experiments show that the removal of the hypophysis in itself has little effect on the size of the thymus, but a certain degree of thymus involution can occur immediately after hypophysectomy, under the influence of external stimuli. It seems that the secretion by the adrenal of the hormone causing thymus involution is inhibited, but not completely abolished, in the absence of the hypophysis. Since it is only the cortex which shows definite atrophy

Table VII.

Hypophysectomized.	Thymus weight (mg.).						Adrenal weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
24 hrs. after hypophysectomy : 2 months old	207	245	201	303	246	353	37	39	25	43	24	30	126	114	110	106	128	140
Legs tied : 3 months old. All died within 24 hrs.	198	218	180	140	230	148	30	42	32	32	45	33	149	159	139	159	129	136
Formaldehyde : 0·5 c.c. of 4 p.c. solution. 3 months old. All died within 24 hrs.	121	150	132	200	121	130	40	35	36	40	36	36	118	140	118	142	133	152
Adrenaline (1/1000) : 0·2 c.c. twice daily. 3 months old. All died within 24 hrs.	107	215	105	154	130	187	40	44	41	50	39	46	119	145	145	150	139	137
Fasted 96 hrs. 5 months old	101	119	94	104	90	87	39	37	41	40	37	39	175	183	175	178	169	132

Table VIII.

Hypophysectomized + saline.	Thymus weight (mg.).						Adrenal weight (mg.).						Body weight (g.).					
	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.	1.	2.	3.	4.	5.	6.
Legs ties : 2½ months old. Died within 36 hrs.	297	201	335	322	219	228	44	24	24	34	32	32	107	114	124	116	113	106
Formaldehyde : 0·5 c.c. twice daily. Died within 36 hrs.	270	228	160	254	150	261	52	38	38	42	40	32	158	152	140	173	144	166
Adrenaline : 0·2 c.c. twice daily. Died after 24 hrs. 2½ months old	186	277	150	186	240	276	31	36	42	44	30	52	117	134	108	123	116	126
Hypophysectomized + cortin.																		
Adrenaline : 0·2 c.c. twice daily + cortin 0·2 c.c. 3 times per day, 48 hrs. 5 months old	120	110	176	98	125	135	41	33	31	32	40	34	159	180	160	150	129	115
Formaldehyde : 0·5 c.c. of 4 p.c. solution twice daily + cortin 0·2 c.c. 3 times per day, 48 hrs. 5 months old	137	99	110	138	81	89	50	40	41	42	38	40	165	167	176	145	170	160

after hypophysectomy, one would think that the cortex was essential for the involution of the thymus; but if it is recalled that surgical removal of the medulla will also inhibit thymus involution—even though a sufficient amount of cortical tissue is left intact—it would seem that both the cortex and the medulla have to be active in order to allow of thymus involution.

DISCUSSION.

The question arises: what is the probable significance of the reaction of the thymus and the adrenal to damaging influences?; for it cannot be mere coincidence that the adrenal and the thymus react first in such cases. The extensive studies made on the problem of excitement hyperglycæmia and the so-called "Fesselungs Hyperglykämie" of the German authors have undoubtedly showed already that adrenaline secretion occurs very easily even after mere psychic excitement. The fact that adrenaline secretion is stimulated by toxic doses of various drugs and that the processes of detoxification are interfered with in the absence of the adrenal have also long been known. If we compare our own findings with those described in the literature, we come to the conclusion that whenever the organism is placed in a critical situation it meets it with a typical defence reaction. This defence reaction consists of loss of body-weight, adrenal enlargement combined with loss of cortical lipoids, thymus œdema often combined with the formation of pleural transudate and sometimes retroperitoneal œdema. All these changes in the adrenal, the thymus, and water metabolism are to a certain degree under pituitary control because the pituitary stimuli are necessary to maintain the adrenal in a fully functional condition, while the adrenal, in turn, is essential in order to allow the thymus to respond. This defence reaction is most marked when a damaging influence acts on the organism for the first time. We saw repeatedly that, after an initial enlargement, the adrenal decreases in size and returns to normal even though the injections of the drug (such as morphine, atropine or adrenaline) which produced the initial enlargement are continued. In such cases the lipoid content of the cortex also returns to normal and the thymus resumes its normal size. It seems, therefore, that the changes in these glands are not due simply to the presence of the toxic substance in the organism, but are the morphological expression of an "alarm reaction" which enables the organism to meet critical situations efficiently. The significance of the changes in water metabolism, particularly of the tendency toward œdema formation, is not quite clear. The frequent occurrence of thymus œdema and the formation of pleural transudates show, however, that changes in water metabolism do occur during this alarm reaction of the organism, and we should like to mention in this connection that subcutaneous injection of formalin in the dose we used caused marked subcutaneous œdema in our adrenalectomized animals, while no such effect was seen in the other groups. The clinical conditions of drug habituation on the one hand, and the so-called toxic œdema and hunger œdema on the other, may have some bearing on this problem. Furthermore, it seems possible that unspecific fever therapy and protein therapy act by eliciting such an alarm reaction, which would help the organism to defend itself even against stimuli other than those which

elicited the reaction. We should like to point out that among all the damaging influences—toxic substances, surgical shock, exposure to cold, hunger, etc.—none failed to produce adrenal cortical hypertrophy if the experimental conditions damaged the animal seriously enough. This point is particularly important in view of the fact that many experimenters assay their pituitary preparations for adrenotropic activity on non-hypophysectomized animals. We must consider this method as unspecific, on the basis of the experiments mentioned in this paper, for any toxic extract will lead to cortical hyperplasia. Significant assays can only be made on hypophysectomized animals (Collip, Anderson and Thomson, 1933).

SUMMARY.

1. A syndrome consisting of rapid involution of the thymus, formation of a pleural transudate, and adrenal enlargement with loss of cortical lipoids, is produced in the rat by certain operative injuries, drugs, exposure to low temperature, and stimuli merely causing nervous excitement. In fact, among all the stimuli tried, none has been found which would seriously damage the experimental animal without causing rapid thymus involution and adrenal enlargement. Involution produced by such means is more pronounced than that which follows withdrawal of all food and water, and is therefore not due to failure of the animal to eat.

2. All the injuries and drugs which normally cause thymus involution are without effect on the size of the thymus of adrenalectomized animals. In hypophysectomized animals they have some effect, but much less than in normals.

3. A method for the isolated removal of the adrenal medulla with a suction pump is described. This method allows of removal of the medulla without seriously injuring the cortex. The thymus of rats thus deprived of medullary tissue but still in possession of active cortical tissue shows little if any ability to involute.

4. The inability of the thymus to undergo involution in adrenalectomized animals is not restored by sodium chloride, cortin in such doses as have been used, or adrenaline treatment.

5. Sodium chloride restores the otherwise low resistance of adrenalectomized rats to toxic doses of various drugs if the animals receive food. It is quite inactive, however, in the fasting animal.

6. The changes caused by a drug when it is given for the first time will subside later in spite of the continued administration of this drug. It appears, therefore, that the syndrome which we described consisting of thymus œdema followed by involution, formation of a pleural transudate, enlargement of the adrenal cortex with loss of its lipoid granules, and the accompanying rapid loss of body-weight, represent an "alarm reaction" which enables the organism to meet critical situations more efficiently. Attention is called to the possible connections between these experimental findings, and the clinical observations of drug habituation on the one hand and non-specific fever and protein therapy on the other hand.

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REFERENCES.

BOINET, E.—(1899) *C.R. Soc. Biol.*, Paris, **51**, 671, 673.
COLLIP, J. B., ANDERSON, E. M., AND THOMSON, D. L.—(1933) *Lancet*, **2**, 347.
DUSTIN, A. P.—(1921) *C.R. Soc. Biol.*, Paris, **85**, 260, 1103.
GAUNT, R., TOBIN, C. E., AND GAUNT, J. H.—(1934) *Proc. Soc. exp. Biol.* N.Y., **32**, 134.
JAFFE, H. L.—(1924) *J. exp. Med.*, **40**, 619.
LELOIR, L. F., AND NOVELLI, A.—(1933) *C.R. Soc. Biol.*, Paris, **114**, 798.
MARINE, D., MANLEY, O. T., AND BAUMAN, E. J.—(1924) *J. exp. Med.*, **40**, 429.
PERLA, D., AND MARMORSTON-GOTTESMAN, J.—(1931) *Proc. Soc. exp. Biol.*, N.Y., **28**, 475.
STAR, P.—(1895) *Lancet*, **1**, 284.
v. SPRETER, T.—(1935) *Z. ges. exp. Med.*, **96**, 95.